Investigating polyol and acetic acid metabolism in wine related non-*Saccharomyces* yeasts

by

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Declaration

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Summary

Glycerol is the main polyol produced in *Saccharomyces cerevisiae* not only to counterbalance osmotic pressure but also to adjust redox balance. Incidentally, it may also contribute to the smooth mouthfeel of wine. Whereas glycerol is closely linked to acetic acid production in *S. cerevisiae*, this correlation is not as clear in non-*Saccharomyces* yeasts (particularly *Torulaspora delbrueckii*).

Additional polyols - which function as stress protectants and could potentially influence wine mouthfeel - have been reported in wine but the producing yeasts were never isolated. *Lachancea thermotolerans*, *Starmerella bacillaris* and *T. debrueckii* have been recently described as producing other polyols in addition to glycerol with the latter producing the highest amounts. However, the enzyme assays used were limited to polyol detection in combination.

Thus, the aim of this study was to optimize chromatography-based methods for the separation of polyols and to investigate the production of these compounds in non-*Saccharomyces* yeasts under a variety of environmental conditions.

Gas Chromatography-Mass Spectrophotometry was successful for the separation of polyols but only in fermentation samples with no residual sugars. Since non-*Saccharomyces* yeasts do not ferment to completion, other methods are required for the individual detection of polyols in order to follow production throughout fermentation.

Our data show that in addition to glycerol, three *T. delbrueckii* strains increasingly produced similar amounts of D-sorbitol, D-arabitol and D-mannitol throughout fermentation. Furthermore with the exception of glycerol, *T. delbrueckii* produced higher amounts of polyols in grape must when compared to synthetic must. Whereas glycerol is limited to NADH recycling, these additional polyols may increase the co-factor recycling pool in *T. delbrueckii*.

Our data also show that D-sorbitol, D-mannitol and D-arabitol production was influenced by initial sugar concentration with the highest amounts detected for D-arabitol in *T. delbrueckii*. In contrast to D-arabitol which was produced at the highest amounts, D-mannitol and D-sorbitol were not induced by NaCl. It is possible that these compounds may have accumulated within the cell as a consequence of the osmotic gradients or mechanisms related to the prevention of ion toxicity as observed in literature.

Polyol production was repressed in acetic acid media in this study and induced in ethanol supplemented media. The intake of acetic acid could have resulted in a change in redox balance and a reduced need for polyols as reported in literature. The presence of ethanol could have resulted in readjustment of polyol retention within the cell and release of polyols.

Overall this study shows that non-*Saccharomyces* yeasts (particularly *T. delbrueckii*) are capable of polyol production. The amounts of polyols produced in some non-*Saccharomyces* yeasts may have a direct impact on wine but further investigations are required on this.

Opsomming

Gliserol is die hoof poli-ol wat deur *Saccharomyces cerevisiae* geproduseer word, nie net om osmotiese druk teen te werk nie, maar ook om die redoksbalans aan te pas. Dit mag ook bydrae tot die gladde mondgevoel van wyn. Waar gliserol baie nou geskakel is met die asynsuur produksie in *S. cerevisiae*, is hierdie korrelasie nie so duidelik in nie- *Saccharomyces* giste (veral *Torulaspora delbrueckii*) nie. Ander poli-ole – wat optree as spanningsbeskermers en moontlik wyn mondgevoel kan verander- is voorheen geraporteer in wyn, maar die produserende giste is nooit ge-isoleer nie. *Lachancea thermotolerans, Starmerella bacillaris* en *T.delbrueckii* is onlangs beskryf as produsente van poli-ole anders as gliserol, met die laasgenoemde wat die hoogste aantal produseer. Alhoewel die ensiem toets wat gebruik is slegs poli-ole in kombinasie kon optel.

Die doel van hierdie studie was om die chromatograaf-gebaseerde metode te optimiseer vir die skeiding van poli-ole en om die produksie van hierdie verbindings in nie-*Saccharomyces* giste onder ‘n variasie van omgewingstoestande te toets.

Gas chromotograaf-massa spektrofotometrie was suksesvol vir die skeiding van poli-ole, maar slegs in monsters van fermentasies wat geen residuele suiker bevat nie. Aangesien nie-*Saccharomyces* giste nie tot droogheid fermenteer nie, word ander metodes benodig vir die individuele deteksie van poli-ole om die produksie gedurende fermentasie te volg.

Ons data toon dat addisioneel tot gliserol, drie *T.delbrueckii* rasse toenemend soortgelyke konsentrasies van D-sorbitol, D-arabitol, en D-mannitol geproduseer het gedurende fermentasie.

Met die uitsondering van gliserol, produseer *T.delbrueckii* ‘n hoër aantal van poli-ole in druiwe sap as in sintetiese mos. Waar gliserol beperk is tot NADH herwinning, mag hierdie ander poli-ole die ko-faktor herwinnings poel in *T.delbrueckii* verhoog. Die data wys ook dat D-sorbitol, D-mannitol en D-arabitol produksie beïnvloed word deur die oorspronklike suikerkonsentrasie, met die hoogste konsentrasie gevind vir D-arabitol in *T. delbrueckii* fermentasies.

In kontras met D-arabitol wat in die hoogtse konsentrasies geproduseer word, is D-mannitol en D-sorbitol produksie nie deur NaCl ge-induseer nie. Dit is moontlik dat hierdie verbindings in die sel geakkumuleer het as ‘n nagevolg van die osmotiese gradient of meganismes verwand aan die voorkoming van ion vergiftiging soos in die literatuur bespreek.

Poli-ool produksie was onderdruk in asynsuur media in hierdie studie en aangewakker in etanol aangevulde media. Die inname van asynsuur kon ‘n verandering in die redoksbalans tot gevolg gehad het en die en ‘n verlaging in die behoefte vir poli-ole soos in die literatuur bespreek. Die teenwoordigheid van etanol was moontlik verantwoordelik vir die aanpassing in die poli-ool retensie binne-in die sel en die vrystelling van poli-ool.

Hierdie studie wys dat nie-*Saccharomyces* giste (veral *T.delbrueckii*) in staat is tot poli-ool produksie. Die aantal poli-ole wat deur sommige nie-*Saccharomyces* giste geproduseer word het moontlik ‘n direkte impak op wyn, maar verdere ondersoeke word benodig.

This thesis is dedicated to my family for their love and support.

Biographical sketch

Lethiwe Lynett Mbuyane was born on the 5th of October 1993 in Limpopo, South Africa. She matriculated from Calvin College, Burgersfort in 2011 and obtained a BSc-degree (Molecular and Life Sciences) in 2014 from the University of Limpopo. Lethiwe obtained an Hons-BSc degree in Wine Biotechnology in 2015 and commenced with a MSc in Wine Biotechnology at Stellenbosch University.

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Preface

This thesis is presented as a compilation of 4 chapters.

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General introduction and project aims

Chapter 1: General introduction and project aims

1.1 Introduction

Wine results from the biochemical conversion of grape must sugars into ethanol (as well as other by-products) within an environment comprising yeasts and bacteria possessing different fermentation capabilities. In literature, it has been generally reported that non-*Saccharomyces* yeasts dominate at the beginning of a spontaneous fermentation. However, as fermentation continues and growth conditions become sub-optimal (because of oxygen depletion, decreased nutrient levels, increasing ethanol and acetic acid concentrations etc.), most of the yeasts belonging to this group decline and *Saccharomyces cerevisiae* takes over the fermentation to completion (Capozzi et al. 2015). Thus, *S. cerevisiae* strains have been selected and commercialised for conventional use in wineries as they allow for highly efficient and reproducible fermentations (Contreras et al. 2015; Wang et al. 2016). Nevertheless, despite their weaker fermentation performances, non-*Saccharomyces* yeasts are still valuable as they can add their own oenological footprint and bring about organoleptic complexity to the wines. Recently, interest has therefore shifted towards the use of non-*Saccharomyces* yeasts in multi-starter and sequential fermentations in an attempt to modify wine flavour while reducing the risk of a stuck fermentation (Soden et al. 2000; Jolly et al. 2014; Wang et al. 2016).

Since non-*Saccharomyces* yeasts have been reported to be most active at the early stages of spontaneous fermentation, it is important to understand how these yeasts respond to stresses to which they are exposed in grape juice. As a consequence of high sugar concentrations characteristic of grape must, osmotic stress is most prevalent at the beginning of fermentation. When the yeast cell is inoculated into/exposed to grape must with high sugar levels, there is an imbalance between the intra- and extracellular solute environment. The osmotic gradient causes a change in water movement along the cell membrane and regulatory mechanisms are required to prevent water loss and cell death (Hohmann 2002; Li et al. 2010).

Osmoregulatory mechanisms include the use of salts, ions and sugar alcohols in an attempt to maintain turgor pressure as well as the functioning of biological activities during osmotic stress. Sugar alcohols (also referred to as polyols) are a class of carbohydrates whose carbonyl group (aldehyde or ketone) has been reduced to a primary or secondary alcohol (Moon et al. 2010). Glycerol is a well-known sugar alcohol that has been extensively researched as a compatible solute regulated by the High Osmolarity Glycerol (HOG) pathway in *S. cerevisiae*. In addition to protecting the cell in high solute environments, glycerol is also produced to address redox imbalance caused by surplus NADH generated from biosynthetic reactions (Hohmann 2002; Noti et al. 2015).

In *S. cerevisiae*, the production of glycerol is associated with increased levels of acetic acid as a consequence of redox balance (Eglinton et al. 2002; Rantsiou et al. 2012; Noti et al. 2015). However, the link between glycerol and acetic acid is not as clear in some non-*Saccharomyces*

yeasts in comparison to *S. cerevisiae*. In particular, fermentations involving yeast species such as *Starmerella bacillaris*, *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima* and *Pichia kluyveri* result in wine with a low final volatile acidity (Rantsiou et al. 2012; Capozzi et al. 2015; Wang et al. 2016). Furthermore, a disconnect between the amount of sugar consumed in *T. delbrueckii* and the levels of glycerol as well as acetic acid produced was observed. This yeast was also reported to ferment well and produce low levels of by-products (involved in redox balance) but the mechanisms behind this are unknown (Renault et al. 2009).

In addition to glycerol, polyols such as erythritol, mannitol, arabitol and sorbitol have been detected in wine. However, the producing microorganisms have never been isolated and it was assumed that bacteria, yeast or fungi were responsible (Margalit 2012). Indeed, yeast species such as *Zygosaccharomyces* and *Candida* synthesize mannitol, erythritol and arabitol with functions related to osmotic, redox and heat stress protection (Yu et al. 2006; Saha et al. 2007). Recently, we have shown that *L. thermotolerans*, *St. bacillaris* and *T. delbrueckii* produce fairly high concentrations of mannitol/arabitol and sorbitol/xylitol in addition to glycerol (De Kock 2015). The latter author also noticed low acetic acid levels in these yeasts during alcoholic fermentation. Studies which focus on sugar alcohol production in wine-related yeast are limited and the mechanisms behind the synthesis of these compounds while maintaining low volatile acidity are mostly unknown. Therefore it is important to characterise the behaviour of specific non-*Saccharomyces* yeasts under unfavourable conditions characteristic of alcoholic fermentation (osmotic stress, redox imbalances, ethanol, acetic acid accumulation etc.) in terms of polyol production and to determine the role of these compounds under the aforementioned conditions. Since polyols such as xylitol, mannitol and sorbitol were reported to impart a sweet mouthfeel (Zhang et al. 2013; Kordowska-Wiater 2015) to a range of products, the organoleptic impact polyols produced in wine by selected non-*Saccharomyces y*easts also needs to be investigated.

1.2 Rationale and aims

The production of sugar alcohols in non-*Saccharomyces* yeasts during alcoholic fermentation has not been thoroughly investigated. Although a study performed at the IWBT (De Kock, 2015) indicated that selected non-*Saccharomyces* yeasts were capable of producing D-mannitol/L-arabitol and D-sorbitol/xylitol, these observations were only made at the end of fermentation. Furthermore, the enzyme assays used could only detect polyols in combination. Thus it was required to optimize published methods for the individual detection of polyols in fermentation samples. Given the potential oenological roles that these compounds may play on wine mouthfeel and protective roles they may confer to yeasts, this study aimed to investigate the production of sugar alcohols in wine-related non-*Saccharomyces* yeasts under different environmental conditions.

The specific objectives of the study were as follows:

1) Investigate chromatography-based methods for the identification and quantification of polyols
2) Screen selected non-*Saccharomyces* yeasts for polyol and acetic acid production
3) Determine the impact of different environmental conditions on polyol production

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Chapter 2

Literature review: Polyol and acetic acid metabolism in non-*Saccharomyces* yeasts

Chapter 2: Polyol and acetic acid metabolism in non-*Saccharomyces* yeasts

2.1 Introduction

Wine results from the fermentation of grape juice which involves the biochemical conversion of sugars into ethanol and carbon dioxide along with a variety of metabolites. This transformation mainly relies on yeasts, particularly strains of *Saccharomyces cerevisiae*. In a spontaneous fermentation, the early stages are dominated by apiculate yeasts such as *Hanseniaspora/Kloeckera* followed by species belonging to the genera *Metschnikowia*, *Pichia, Kluyveromyces*, *Schizosaccharomyces*, *Candida*, *Starmerella*, *Torulaspora*, *Rhodotorula* and *Zygosaccharomyces* among others (Gobbi et al. 2013; Englezos et al. 2015). However, as conditions become limiting due to nutrient limitation combined with oxygen depletion, increasing concentrations of ethanol, acetic acid and a few other inhibiting compounds, these populations decline and *S. cerevisiae* takes over the fermentation to completion. Therefore, several strains of *S. cerevisiae* have been selected and commercialised for the wine industry on the basis of their ability to ferment in a highly efficient, controllable and reproducible manner (Jolly et al. 2003; Ciani et al. 2010). In an attempt to introduce oenological complexity and modify wine flavour profiles while limiting the risk of an unpredictable fermentation, winemakers have used unconventional strains with *S. cerevisiae* in multi-starter co- or sequential fermentations (van Breda et al. 2013; Renault et al. 2015; Padilla et al. 2016). While indigenous yeasts are important for their metabolic activities, not much is known about their behaviour during alcoholic fermentation. Thus, research into evaluating how non-*Saccharomyces* yeasts adapt to the environmental conditions pertaining to grape juice and those occurring during alcoholic fermentation is being conducted (Renault et al. 2015; Padilla et al. 2016). In particular, their adaptation to osmotic stress has been the focus of various recent studies. Indeed, osmotic stress is most prevalent at the early stages of a fermentation and glycerol is a well-known compatible solute produced in *S. cerevisiae*. However, the synthesis of glycerol in *S. cerevisiae* has been linked to increased levels of acetic acid due to the regulation of redox balance especially in high sugar musts (Li et al. 2010; Munna et al. 2015; Noti et al. 2015). Unlike glycerol which imparts smoothness and sweetness to wine, acetic acid is the main constituent of wine volatile acidity giving off a vinegary aroma at high levels. Thus strains that are capable of producing moderate amounts of glycerol and acetic acid are desired in winemaking. Indeed selected non-*Saccharomyces* yeasts have been observed to behave differently by producing varying amounts of glycerol and low amounts of acetic acid (Bely et al. 2008; Renault et al. 2009; van Breda et al. 2013). Furthermore, a variety of polyols have been detected in wine but the producing strains were never isolated and it was assumed that bacteria, yeasts and fungi may be responsible (Margalit 2012). Indeed, selected non-*Saccharomyces* yeasts were reported to produce polyols other than glycerol under a variety of conditions (Zhu et al. 2010;

De Kock 2015; Stincone et al. 2015). Since these compounds (i.e. polyols and acetic acid) may impact the wine's organoleptic properties, investigating their production is not only scientifically engaging but also practically relevant. In this review, the metabolic routes responsible for the synthesis of selected sugar alcohols by non-*Saccharomyces* yeasts will be discussed. As glycerol is linked to acetic acid production during alcoholic fermentation, factors influencing acetic acid metabolism will also be reported on.

2.2 Pathways involved in polyol production

2.2.1 The function and distribution of sugar alcohols throughout nature

Polyols are widely distributed throughout nature and are found in plants, animals and microorganisms. The physiological functions have been related to carbon storage, reductant recycling, efficient carbon fixation and as compatible solutes in stressful environments (Jeya et al. 2009). Polyols have also been isolated from fungal spores serving as storage compounds and have been reported to be involved in pathogenicity by providing means for infectious microorganisms to store carbohydrates as well as reduce energy in a form that is not available for the host (Voegele et al. 2005). It was also observed that soil amendment with selected sugar alcohols increases microbial and enzyme activity (Yu et al. 2016). In wine yeasts, glycerol is a well-known sugar alcohol that is not only synthesized as an integral part of central carbon metabolism but also as a compatible solute functioning to relieve osmotic stress and counteract redox imbalance in a cell (Noti et al. 2015).

2.2.2 High Osmolarity Glycerol pathway

As a yeast cell is inoculated into or encounters the grape must environment, the high sugar levels cause an imbalance between the intra- and extracellular solute environment resulting in a condition known as osmotic stress (Mager and Siderius 2002; Hernandez-Lopez et al. 2003; Noti et al. 2015). This imbalance results in an osmotic gradient which causes a change in water movement along the cell membrane and water is lost from the cell. If regulatory mechanisms are not put in place to prevent this action, the cell will eventually shrivel up and die. Osmoregulatory mechanisms that come into play in such conditions include the use of salts, ions and carbohydrates (such as polyols) to maintain turgor as well as the functioning of biological activities. Glycerol has been extensively researched as a compatible solute produced in *S. cerevisiae* during alcoholic fermentation (Hohmann 2002). In conditions of stress, this sugar alcohol is synthesized through the High Osmolarity Glycerol (HOG) pathway mediated by

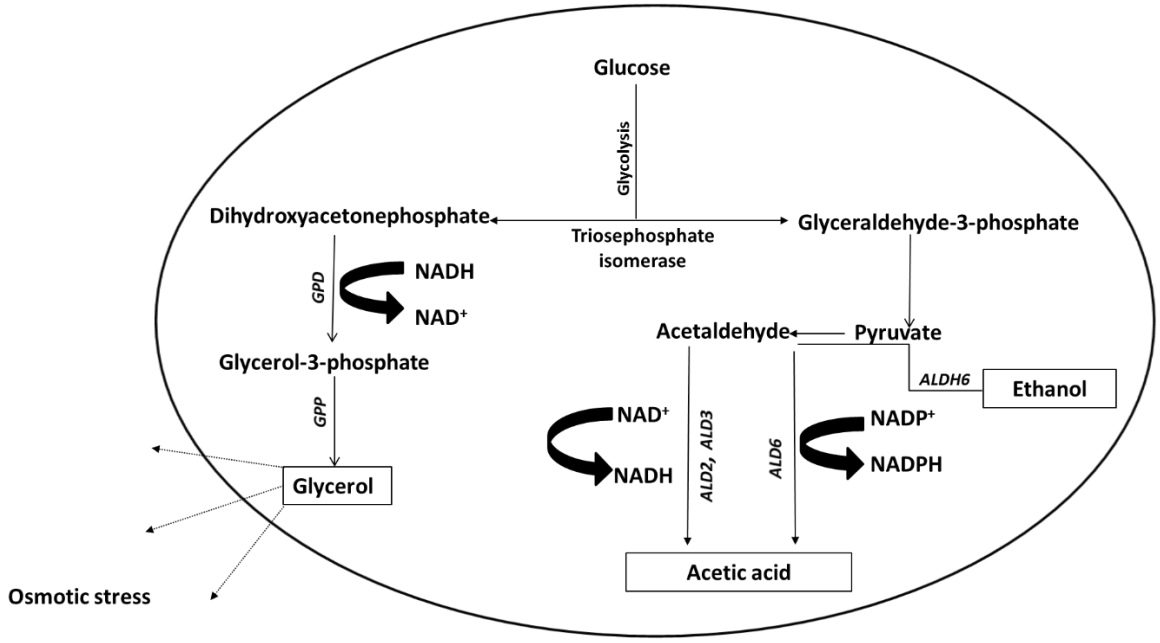

Figure 1: Glycerol and acetic acid production for counteracting osmotic stress and redox imbalances during alcoholic fermentation. *GPD1*-glyceraldehyde dehydrogenase; *GPP*-glyceraldehyde phosphatase; *ALD*-aldehyde dehydrogenase; *ALHD*-alcohol dehydrogenase.

a Mitogen Activated Protein Kinase (MAPK) signalling system. In high solute concentrations, the cell detects a change in the environment via two osmosensors: Sln1p and Sho1p. This results in the activation and rapid accumulation of kinase *Hog1p* which in turn leads to the expression of genes involved in glycerol production (O'Rourke et al. 2002). Figure 1 summarises the steps involved in glycerol synthesis from the glycolytic intermediate dihydroxyacetone phosphate in a two-step catalytic reaction involving the enzymes glycerol-3-phosphate dehydrogenase (Gpdp) and glycerol-3-phosphatase (Gppp). Each step of the glycerol production pathway is catalysed by two isoenzymes. *GPD1* is expressed under hyperosmotic stress whereas *GPD2* increases in expression under anaerobic conditions. Regarding the glycerol-3-phosphatase, *GPP1* is involved in osmoadaptation and growth whereas *GPP2* is only important for osmoadaptation in anaerobic conditions (Dakal et al. 2014). In addition to protecting the cell from water loss during osmotic stress, glycerol is also produced to protect the cell from redox imbalances. Although the production of ethanol from glucose is redox neutral, surplus NADH generated from biosynthetic reactions cannot be processed through the electron transport chain and the synthesis of glycerol is important for the recycling of this cofactor during alcoholic fermentation (Erasmus et al. 2004). Following molecular responses to a hyperosmotic environment which lead to glycerol accumulation, the cell swells resulting in the inactivation of Sln1p. This leads to the inactivation of the HOG cascade and release of glycerol through aquaglyceroporin Fsp1p into the environment (Hernandez-Lopez et al. 2006).

2.2.3 The Pentose Phosphate Pathway for polyol production

In yeast, glycerol is important when the cells experience osmotic stress, redox imbalances as well as heat stress. However, as indicated in Figure 2, other polyols can be synthesized through the pentose phosphate pathway (PPP). This pathway, found in fungi, mammals and plants, is required for energy generation via the production of NAD(P)H. Additionally, the PPP is important for nucleotide production and amino acid biosynthesis through precursors such as D-ribose-5-phosphate and D-erythrose-4-phosphate. Ribulose-5-phosphate serves as the main intermediate required for polyol production as it can be converted into either ribulose or xylulose-5-phosphate and these intermediates can be transformed into arabitol or ribitol. Figure

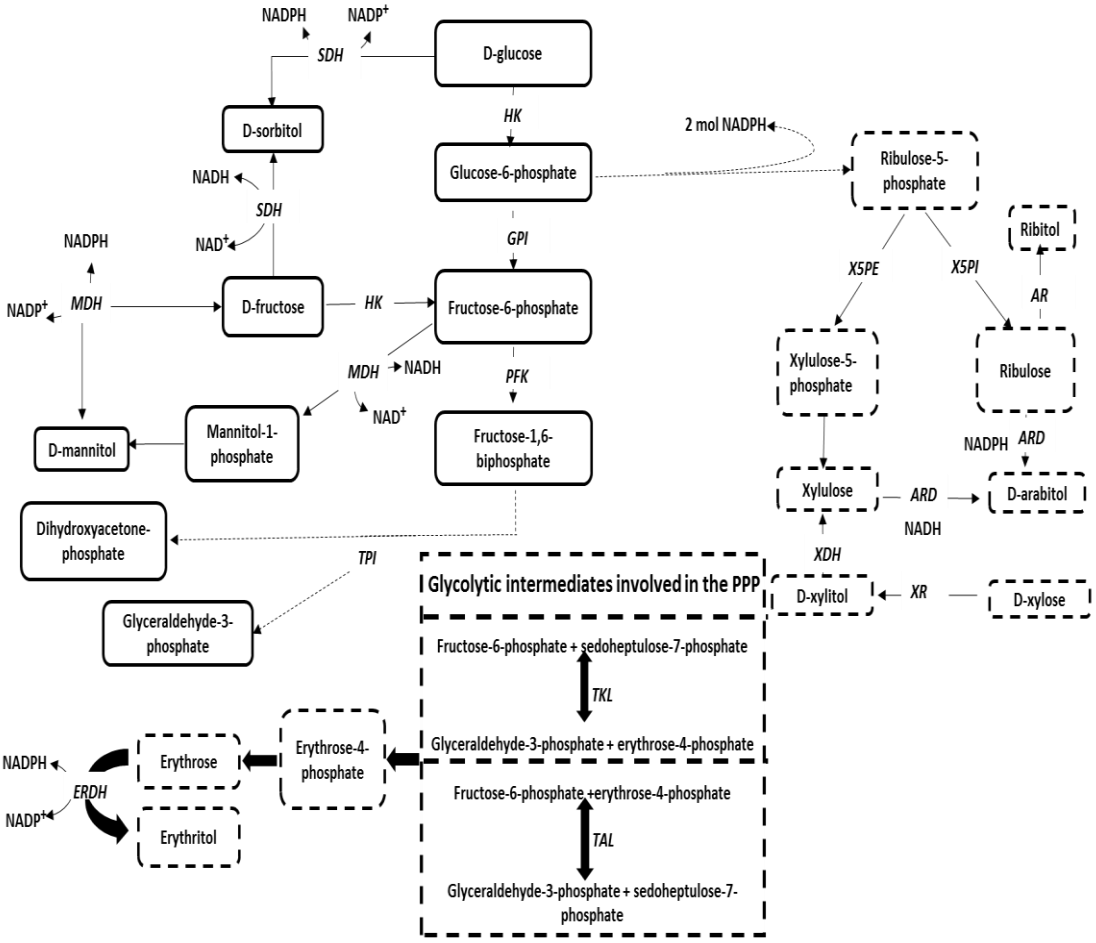

Figure 2: The pentose phosphate pathway and other metabolic routes for polyol production with glucose and fructose as a carbon source. *HK*-hexokinase; *GPI*-glucose phosphate isomerase; *PFK*-phosphofructokinase; *HK*-hexokinase; *MDH* mannitol dehydrogenase; *X5PE*-xylulose-5-phosphate epimerase; *X5PI*-xylulose-5-phosphate isomerase; *EK*-erythrose kinase; *ER*D-erythrose dehydrogenase; *SDH*-sorbitol dehydrogenase; *AR*-aldose reductase; *ARD*-arabitol dehydrogenase; *XR*-xylose reductase; *TAL*-transaldolase; *TKL*-transketolase.

2 indicates how erythritol is produced in a different set of reactions which connect the PPP to glycolysis by sharing intermediates in a set of reversible reactions mediated by transketolase (TKL) and transaldolase (TAL). D-xylitol is a valuable sugar alcohol that is also an intermediate of the PPP but is not a product of the glycolytic cycle. The polyol is produced with D-xylose as

substrate with NAD(P)H linked xylose reductase but can be fed into the PPP by conversion into D-xylulose with NAD^+ requiring xylitol dehydrogenase (Lin et al. 2001; De Muynck et al. 2006; Saha et al. 2007; Moon et al. 2010; Kordowska-Wiater 2015; Stincone et al. 2015).

2.3 Polyol production in yeast under non-wine related conditions

2.3.1 Yeasts producing polyols through the Pentose Phosphate Pathway

While glycerol is produced as the main polyol in most yeast species, the PPP is responsible for the synthesis of D-arabitol, ribitol and erythritol in yeasts (Table 1). Some yeasts are capable of synthesizing a specific polyol from different metabolic routes. This has been observed in yeasts that are capable of producing D-arabitol from either the xylulose or ribulose forming part of the PPP (Figure 2). *Saccharomyces mellis*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii* along with selected yeasts from the *Pichia*, *Hansenula* and *Candida* genera were reported to produce D-arabitol via the reduction of D-ribulose with an NADP-dependent pentitol dehydrogenase (Ahmed 2001; Zhu et al. 2010; Kumdam et al. 2013). A strain of *Z. rouxii* was also observed to synthesize D-arabitol in an alternate route with D-xylulose as a substrate with an NAD-dependent polyol dehydrogenase (Wong et al. 1995). The metabolic routes for arabitol production are not always clear and require further investigation as was observed in *Candida albicans* whereby a mutant lacking the arabitol dehydrogenase gene was still able to synthesize the sugar alcohol with glucose as substrate (Wong et al. 1995; Kayingo and Wong 2005). Furthermore, some non-*Saccharomyces* do not produce a single polyol, instead a mixture is synthesized. Whereas glycerol is mostly produced as the main polyol in most yeasts, additional polyols are produced depending on the strain and cultivation conditions used (Table 1). Indeed *H. anomala* was reported to produce arabitol in addition to glycerol. As expected, glycerol was observed as the main compatible solute but the function of arabitol was less clear and it was assumed that the polyol may serve as a secondary solute when glycerol is consumed (Van Eck et al. 1989). Studies focused on erythritol production have mostly been based on the reduction of erythrose. However, erythritol has also been isolated in fructophilic *Candida magnolia* along with *Yarrowia lipolytica*, *Pseudomyzoma tsukibaensis* and *Torula corallina* with glucose as a substrate (Lee et al. 2002; Yu et al. 2006; Lin et al. 2010; Kim et al. 2013). Although the polyols mentioned here are limited to the PPP, yeasts are capable of synthesizing polyols through other metabolic routes.

2.3.2 The production of sugar alcohols from other metabolic routes

2.3.2.1 D-Mannitol

Mannitol can be produced via fructose-6-phosphate and mannitol-1-phosphate (as seen in Figure 2) through a consecutive catalytic reaction mediated by a NAD^+ or $NADP^+$ dependent

dehydrogenase (Lee et al. 2003a; Voegele et al. 2005). Two mannitol dehydrogenase (*MDH*) open reading frames have been reported for *S. cerevisiae* and one of these open reading frames were overexpressed in a mutant unable to synthesize glycerol. Mannitol was reported to confer resistance to salt stress (1.5 M NaCl) and heat stress up to 50°C (Watanabe et al. 2006). The industrial production of mannitol was investigated in a strain of *Candida magnoliae* isolated from fermentation lees with glucose and fructose as substrates (Song et al. 2002; Lee et al. 2003b). Furthermore, *Torulopsis versatilis*, *Torulopsis anomala*, *Torulopsis nodaensis* and *C. neoformans* were also observed to synthesize mannitol in addition to glycerol (Onishi and Suzuki 1968).

Table 1: Polyol production and acetic acid metabolism of yeasts.

Yeast	Sugar alcohols	Acetic acid		References
		Production	Consumption	
Saccharomyces cerevisiae	Glycerol	✓	✓	Wong et al. 2006; Watanabe et al. 2006; Vilela-Moura et al. 2008
Torulopsis. versatilis, T. pinus, T. nodaensis	Glycerol, mannitol, sorbitol	✓	?	Yonehara and Tani 1987
Candida succiphila, C. boidini, C. magnolia, C. albicans, C. guilliermondii	Glycerol, xylitol, sorbitol, ribitol, arabitol	✓	✓	Ko et al. 2006; Wong et al. 1995
Hansenula anomala, H. polymorpha	Glycerol, arabitol,	✓	?	Van Eck et al. 1989; Hollenberg, 1990
Debaromyces napelensis, D. hansenii	Glycerol, arabitol, xylitol	✓	?	Ko et al. 2006; Kumdam et al. 2013
Metschnikowia pulcherimma, M. reukafii	Glycerol, arabitol	✓	?	Nozaki et al. 2003
Pichia sorbitolphilia, P. kluyveri, P. stipitis, P. fermentas	Glycerol, arabitol, erythritol	✓	✓	Wong et al. 1995; Zhu et al. 2010;
Zygosccharomyces acidifaciens, Z. rouxii, Z. barkeri	Glycerol, Arabitol	✓	✓	Wong et al., 1995
Torulaspora delbrueckii	Glycerol, mannitol/arabitol, sorbitol/xylitol	✓	✓	Casal et al. 2008; Renault et al. 2009; de Kock 2015
Lachancea thermotolerans	Glycerol, mannitol/arabitol, sorbitol/xylitol	✓	✓	Vilela-Moura et al. 2008; Gobbi et al. 2013; de Kock 2015
Starmerella bacillaris	Glycerol, mannitol/arabitol, sorbitol/xylitol	✓	?	Rantsiou et al. 2012; de Kock 2015
Brettanomyces bruxellensis	×	✓	✓	Steensels et al. 2015

Keys: Yes (✓) No (×), Unknown (?)

2.3.2.2 D-Sorbitol

Similar to mannitol, the synthesis of sorbitol is possible with glucose and fructose as substrates. As indicated in Figure 2, sorbitol can arise from fructose in a reversible reaction mediated by NAD^+ dependent sorbitol dehydrogenase or from the reduction of glucose via a NADP

dependent polyol dehydrogenase (Vongsuvanlert and Tani 1988; Silveira and Jonas 2002; Jonas and Silveira 2004). In *gpdΔ* mutants of *S. cerevisiae*, sorbitol and mannitol were observed to function as compatible solutes but the protective effects of these sugar alcohols could not completely substitute those of glycerol (Shen *et al*., 1999). Studies concerning sorbitol production in yeasts are limited and this compound was only detected in methanol-producing yeasts such *Torulopsis pinus*, *Hansenula ofunaensis* along with *Candida succiphila* using glucose as a carbon source with a NAD^+ linked dehydrogenase (Yonehara and Tani 1987). Similarly, an NAD^+-dependent sorbitol dehydrogenase was isolated in *S. cerevisiae* but the enzyme was observed to be induced in sorbitol-containing medium and the role of this polyol as a compatible solute was not explored (Sarthy and Idler 1994).

2.4 Polyol production under wine conditions

2.4.1 Substrates available in grape must for polyol production

The substrate or carbon source available to a yeast determines the kind of polyol/s that may be synthesized. So far, glucose and fructose have been discussed as substrates responsible for the production of polyols resulting from the PPP and other metabolic routes (Figure 2). Table 1 also illustrated the production of polyols in yeast with a variety of substrates under conditions that were mostly not wine related. However, there are a variety of sugars found in grape must which may serve as substrates for sugar alcohol production. Glucose and fructose are the major sugars in grape must and can be found at levels varying from 80 g/l to 130 g/l. Sucrose can be detected in grape juice at 2-10 g/l, L-arabinose at 0.5-1.5 g/l and the maximum amount of D-xylose found was 0.5 g/l. Other sugars detected in grape must are L-rhamnose (0.15-0.4 g/l) and pectin at 0.2-4 g/l (Margalit 2012).

2.4.2 Types of polyols found in wine

2.4.2.1 Glycerol as the main polyol produced by yeasts in wine

Glycerol is produced as an integral part of carbon metabolism in most yeasts species as indicated in Table 1 and is the main polyol found in wine as shown in Table 2. This compound is especially important during alcoholic fermentation as it is produced as a compatible solute in conditions of osmotic stress and is involved in redox balance as fermentation progresses (Noti et al. 2015). The link between glycerol and acetic acid has been thoroughly studied in *S. cerevisiae* but has not been established in all non-*Saccharomyces* yeasts. Selected strains of *L. thermotolerans* and *T. delbrueckii* have been observed to produce similar/higher amounts of glycerol when compared to *S. cerevisiae* while maintaining low acetic acid levels (Gobbi et al. 2013; Wang et al. 2016). *St. bacillaris* strains were also observed to ferment efficiently by producing high levels of glycerol and low levels of acetic acid (Gobbi et al. 2013; Englezos et al.

2015; Wang et al. 2016). *T. delbrueckii* was reported to behave differently from *S. cerevisiae* by synthesising moderate amounts of glycerol regardless of sugar concentration during alcoholic fermentation and it was suggested that glycerol may be required for counteracting osmotic stress while some unknown mechanism may be responsible for maintaining redox balance during alcoholic fermentation (Hernandez-Lopez et al. 2006; Renault et al. 2009).

2.4.2.2 Additional polyols detected in wine

Studies which involve sugar alcohol production under winemaking conditions by yeasts are limited but as indicated in Table 2 polyol production during alcoholic fermentation is possible, especially in *Botrytis cinerea*-affected wines. Although the producing strains for these sugar alcohols have not been isolated, it is assumed that bacteria, wild yeasts and molds (primarily *Botrytis*) are responsible (Margalit 2012). In a more recent study, polyol production was observed in the wine yeasts *L. thermotolerans*, *T. delbrueckii* and *St. bacillaris* (de Kock 2015). Among the 3 species, *T. delbrueckii* produced the highest amounts of polyols. However, the assays used were limited to detecting the sugar alcohols in combination (mannitol/arabitol and sorbitol/xylitol). It is therefore unclear if both polyols were synthesized or if only one compound was detected per assay. Thus, further studies are required to identify sugar alcohols individually and to determine whether these compounds are synthesized throughout fermentation in different species and strains. It is also necessary to determine the role of these compounds during alcoholic fermentation and the impact these compounds could have on wine quality.

Table 2: Sugar alcohols found in table and *Botrytis*-affected wine

Sugar alcohol	Levels found in wine (g/l)
Glycerol	4.5-12 **15-25**
Arabitol	0.1-0.6 (white wine) 0.3-0.11 (red wine) **2.3**
Mannitol	0.1-0.4 **±0.1-1**
Sorbitol	0.05-0.2 **1**
Erythritol	0.03-0.1 **0.05-0.6**
Xylitol	0-0.1
Myo-inositol	0.2-0.7 **+0.7**
2,3 Butandiol	0.3-1.8

Keys: Botrytis-affected wine (Bold font)

2.4.3 Possible functions of polyols during alcoholic fermentation

In a high sugar environment, a compatible solute is required by a yeast cell to ensure that there is a balance between the external and internal environment. As discussed above, glycerol serves this purpose in yeast and regulates redox balances during alcoholic fermentation. Indeed, glycerol is the most abundant polyol in wine (Table 2), but the role of additional sugar alcohols in smaller amounts is less clear. Although the production of an additional polyol may also improve a cell's resistance to high solute conditions, the levels of sugar alcohols found in wine are much lower than those of glycerol and the sole role of these compounds as osmoprotectants is questionable. With regard to the recycling of redox equivalents, the production of glycerol only allows for the regeneration of NAD while that of other sugar alcohols allows for the recycling of NAD(P) making the yeast cell potentially more resistant to redox imbalances. Since sugars other than glucose and fructose do exist in wine (viz. sucrose, xylose, arabinose, rhamnose etc.), some yeast species may possess enzymes that allow for sugar alcohol production from these sugars. In some cases, polyols are produced as precursors for important compounds in yeast. Myo-inositol which is synthesized from glucose via inositol-3-phosphate synthase is a precursor for phosphatidylinositol which is required for the synthesis important compounds such as signalling molecules (Henry et al. 2014). So, the production of these polyols may be important for the synthesis of other important metabolites, signalling molecules or structures within the cell. Nevertheless further investigations are required to determine the actual function of additional polyols in yeast during alcoholic fermentation and the impact these compounds might have on acetic acid production.

2.5. Acetic acid production in yeast during alcoholic fermentation

2.5.1. Metabolic routes responsible for the synthesis of acetic acid

During grape must fermentation the synthesis of glycerol is required for osmotic adjustment and redox balance through NADH recycling but the production acetic acid is necessary to further maintain redox balance as indicated in Figure 1 (Miralles and Serrano 1995; Meaden et al. 1997; Noti et al. 2015). Acetic acid is produced through the pyruvate dehydrogenase *(PDH*) bypass during alcoholic fermentation and in this process, pyruvate decarboxylase (*PDC*) converts pyruvate into acetaldehyde and is oxidized to acetic acid by *ALD* or acetaldehyde dehydrogenase allowing for the recycling of NAD(P) (Hohmann 1991; Hohmann 1993; Remize et al. 2000). Enzymes involved in acetic acid production are expressed under different conditions with a variety of co-factor requirements (Table 3). The synthesis of this compound is not only affected by the redox potential or metabolism of a yeast cell but by a variety of factors which will be discussed below.

Table 3: Enzymes involved in pyruvate dehydrogenase bypass for acetic acid production

Enzymes		Functions, cofactor needs, etc.	References
Pyruvate decarboxylase (*PDC*)	*PDC1*	Mg^{2+}, thiamine, highest expressed with glucose as carbon source	Hohmann 1991; Remize et al., 2000
	PDC2	Mg^{2+}, thiamine, required by *PDC1* and *PDC5* for full expression	Hohmann 1993
	PDC5	Mg^{2+}, thiamine, highly expressed in *PDC1* mutant	Seeboth et al., 1990; Hohmann 1993
	PDC6	Mg^{2+}, thiamine, role unknown	Hohmann 1991; Remize et al., 2000
Acetaldehyde dehydrogenase (*ALD*)	*ALD2/3*	NAD,cytosolic, *ALD3* responsive to salt stress	Mirrales and Serrano, 1995; Remize et al., 2000; Pigeau and Inglis, 2005; Mizuno et al., 2006
	ALD4	K^+,NAD, thiols, mitochondrial, requires growth on ethanol	Dickinson 1994 ; Pigeau and Inglis 2005
	ALD5	NAD, mitochondrial, regulates electron transport chain constituents	Wang et al., 1998
	ALD6	Mg^{2+}, NADP, cytosolic, highest expressed, requireds growth on glucose	Meaden et al., 1997; Mizuno et al., 2006
Acetyl-CoA synthetase (*ACS*)	*ACS1*	ATP, CoA, functional during respiratory and respirofermentative growth	Van den Berg et al., 1996; Jong-gubbels et al. 1997
	ACS2	ATP, CoA, constitutively expressed	Van den Berg et al., 1995; Van den Berg et al., 1996

2.5.2 The impact of environmental factors on acetic acid production

Apart from the metabolism of a yeast cell, the grape must composition has an impact on acetic acid production. In addition to the sugar concentration of grape juice, environmental factors such as vitamins, nitrogen content and pH values below 3.1 or above 4 may increase wine volatile acidity (Vilela-Moura et al. 2010b). The excessive clarification of grape must may remove valuable metabolites and cause nutrient imbalances which may also favour the production of acetic acid (Bely et al. 2005). Controlling volatile acidity is required to produce good quality wine and avoid penalties from regulatory authorities. As a result, a variety of techniques have been investigated with the aim of reducing acetic acid levels in wine.

2.6 Techniques aimed at reducing wine volatile acidity

2.6.1 Mechanical approaches

For winemakers, low acetic acid levels are preferred due to regulations that do not permit concentrations higher than 1.2 g/l in standard wine and 2.1 g/l for ice or botrytis affected wine. Unlike glycerol which is slightly sweet and smooth to the taste, acetic acid gives off a ‘vinegary aroma’ regarded as wine spoilage so winemakers have employed a variety of methods to maintain low volatile acidity as indicated in Table 4 (Vilela-Moura et al. 2008). Mechanical methods include the use of membrane processes such as reverse osmosis and nanofiltration which yield an acid rich permeate that requires costly downstream processing (Vilela-Moura et al. 2011).

Table 4: Techniques aimed at controlling wine volatile acidity

Method	Advantages	Disadvantages	References
Mechanical methods: reverse osmosis and nanofiltration	➢ Efficient ➢ No labour required	➢ Uneconomical ➢ Requires downstream processing	Vilela-Moura et al. 2011
Genetic manipulation of wine yeast strain	➢ Efficiently degrades acetic acid ➢ Produces less acetic acid by directing metabolic flux to other compounds	➢ Acid consumption requires oxygen ➢ May compromise wine quality ➢ GMO's prohibited by the wine industry ➢ Undesirable by-products	Remize et al. 2000; Pigeau and Inglis 2005; Cambon et al. 2006
Non-*Saccharomyces* yeasts in sequential or multistarter fermentations	➢ Economical ➢ Selected strains can be used and yield predictable fermentation	➢ Further investigations required to ensure minimal competition between yeasts	Bely et al. 2008; Rantsiou et al. 2012; Gobbi et al. 2013, Wang et al. 2016
Deacidification through refermentation	➢ Commercial *S. cerevisiae* strains are used i.e. predictable ➢ Improvement in wine quality	➢ Requires clarification of acidic wine prior to treatment ➢ May be uneconomical for industry purposes ➢ Can be unpredictable if unknown flora in grape must is used	Vilela-Moura et al. 2010a, Vilela-Moura et al. 2010b; Gobbi et al. 2013

2.6.2 Biological techniques

Strains of *S. cerevisiae* have been engineered for low acetic acid production through the overexpression of genes such as *GPD1* and acetyl-CoA synthetase (*ACS*) or through the disruption of *ALD4* and *ALD6* (Remize et al. 2000; Pigeau and Inglis 2005; Cambon et al. 2006). Furthermore, a genetically engineered strain of *S. cerevisiae* capable of degrading acetic acid has been constructed but strict regulations in the food industry do not allow for the use of GMOs (Remize et al. 2000). Since the wine industry is moving towards the use of non-*Saccharomyces* yeasts, studies have shown that in addition to enhancing the organoleptic properties of wine, selected strains may also help reduce wine volatile acidity (Bely et al. 2008; Vilela-Moura et al. 2010a; Renault et al. 2015). In particular, strains of *St. bacillaris* were suggested for the reduction of acetic acid during mixed fermentation due to sugar consumption (particularly fructose) which may ultimately lead to a reduction in the osmotic stress imposed on *S. cerevisiae* cells (Rantsiou et al. 2012). *T. delbrueckii* was reported to produce small amounts of undesirable compounds such as acetic acid and has thus been recommended for the fermentation of high sugar musts (Bely et al. 2008; Renault et al. 2009). Wine with high levels of acetic acid can also be treated through a refermentation process that involves the addition of fresh grape must to acidic sterile wine. The method relies on the assumption that yeasts with fermentative capabilities degrade acetic acid during the first 50-100 g/l of sugar consumed in grape must. This approach has been described as efficient and low cost. Additionally, the final

acetic acid levels resulting from this are usually lower than 0.3 g/l but the method carries the risk of a unpredictable fermentation detrimental to wine quality especially if unsterile grape must containing unknown microflora is used (Casal et al. 2008; Vilela-Moura et al. 2008; Vilela-Moura et al. 2011).

2.7. Acetic acid consumption in yeast

2.7.1 Factors influencing acetic acid catabolism

In the presence of glucose the cell is subjected to catabolite repression but upon glucose exhaustion, microorganisms are able to metabolize alternative substrates originating from the initial catabolism of sugars i.e. glycerol, ethanol, pyruvate and acetic acid. These compounds can be metabolized through gluconeogenesis and the tricarboxylic acid (TCA) cycle in the presence of oxygen (Van den Berg and Steensma 1995; Berg et al. 1996; Vilela-Moura et al. 2010a). In addition to the metabolic activity of a yeast, acetic acid consumption is also dependent on acid transport, sugar concentration and the pH of the environment.

2.7.1.1The impact of transport and pH on acetic acid intake

The transport of carboxylic acids can be divided into two groups. Firstly, transport can occur in an energy-independent or passive manner where the acid is taken into the cell by simple or facilitated diffusion through a channel or permease (Casal and Cardoso 1996; Casal et al. 2008). Secondly, the transport of intracellular acetic acid can occur through pumps where the anion form of the acid is extruded into the environment. At a low pH such as that found in wine, acetic acid (pKa < 4.75) is found in its undissociated form and being lipid soluble, passes through the plasma membrane and enters the cell by facilitated diffusion (Orlandi et al. 2013). It was also observed that the facilitated diffusion of acetic acid in its undissociated form occurs through the Fps1p channel in *S. cerevisiae* and that the HOG system enhances acetic acid resistance via the degeneration of this aquaglyceroporin (Piper et al. 2001; Mollapour et al. 2009).

2.7.1.2 The effect of sugar on acetic acid consumption

In *S. cerevisiae*, acetic acid consumption is subject to catabolite repression where the assimilation of alternative carbon sources is inhibited (Wolfe 2005). Thus, *S. cerevisiae* was reported to display diauxic growth where acetic acid is only metabolized after glucose has been completely consumed. A similar pattern of consumption has been observed for *T. delbrueckii, Dekkera anomala* and *Kluyveromyces marxianus* (Casal et al. 2008). In contrast, some commercial *S. cerevisiae* strains were reported to be capable of metabolizing acetic acid in the presence of glucose in wine and grape must under semi-aerobic conditions (Vilela-Moura et al. 2010b; Vilela et al. 2015). This alternative growth pattern is known as biphasic growth and was

also observed in *Z. bailii* and *Schizosaccharomyces pombe*. Such a pattern of consumption is linked to the presence of dicarboxylate transporters which allow for the simultaneous intake of fermentable and non-fermentable carbon sources (Rodrigues et al. 2012).

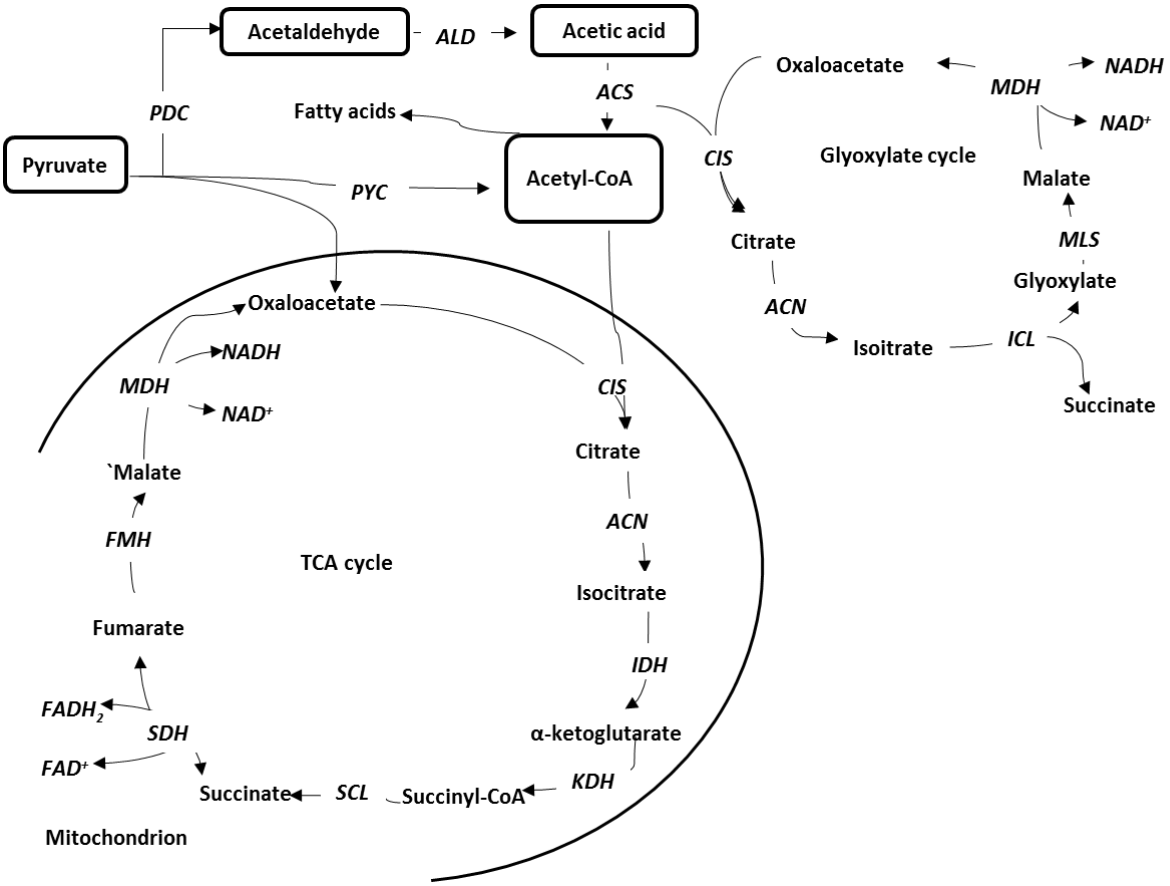

Figure 3: Acetic acid consumption via the TCA and glyoxylate cycle in yeast. *PYC*-pyruvate carboxylase; *PDC*-pyruvate decarboxylase; *ACS*-acetyl-CoA synthetase; *ALD*-aldehyde dehydrogenase; *CIS*-citrate synthase; *CAN*-asconitase; *ICL*-isocitrate lyase; *MLS*-malate synthase; *MDH*-malate dehydrogenase; *KDH*- α-ketoglutarate dehydrogenase; *IDH*-isocitrate dehydrogenase; *SCL*-succinate-CoA ligase; *SDH*-succinate dehydrogenase; *FMH*-fumarate hydratase.

2.7.2 Pathways for acetic acid consumption

Figure 3 indicates how acetic acid is metabolized in the yeast cell. Firstly, acetic acid is broken down to acetyl-CoA in a reaction catalysed by either peroxisomal (Acs1p) or cytosolic (Acs2p) acetyl-CoA synthetase (Jong-gubbels et al. 1997; Dos Santos et al. 2003). The acetyl-CoA arising from this reaction can then be fed into the TCA cycle inside the mitochondria in the presence of glucose. This cycle is responsible for the oxidative generation of NADH, ATP and production of intermediates such as oxaloacetate, succinyl-CoA and *α*-ketoglutarate required for biosynthetic reactions. However, when *S. cerevisiae* is solely grown on a non-fermentable substrate such as acetate, an alternative metabolic route that bypasses oxidative decarboxylation is required for the production of TCA intermediates. This alternative route for acetic acid consumption is known as the glyoxylate pathway and consists of five reactions with three of these being shared with the TCA cycle (Figure 3). Firstly, acetyl-CoA from acetic acid

condenses with oxaloacetate to form citrate via citrate synthase followed by a conversion into isocitrate in a reaction mediated by cytosolic or mitochondrial asconitase. In a reaction specific for this cycle, isocitrate is converted to glyoxylate or succinate by isocitrate lyase. Acetyl-CoA is used up again as it combines with glyoxylate to form malate via malate synthase. As a TCA intermediate, malate is converted to oxaloacetate with NAD^+ linked malate dehydrogenase found in the cytosol. The products of the TCA and glyoxylate cycle from acetic acid consumption are necessary for biosynthetic reactions (Ensign 2006).

2.7.3 Wine related yeasts consuming acetic acid

The consumption of acetic acid requires further investigation especially in a wine context. So far, studies on acetic acid consumption of wine yeast have been mostly based on the 'refermentation approach' of acidic wine in a series of studies involving commercial strains and indigenous yeast isolates. *S*. *cerevisiae* commercial strains and some isolates were screened for the ability to consume glucose and acetic acid under different aeration, glucose and ethanol levels (Vilela-Moura et al. 2008). Selected *S*. *cerevisiae* strains were further evaluated under oenological conditions and were found to be able to consume all glucose and half the amount of acetic acid supplied (Vilela-Moura et al. 2010a). Moreover, it was found that the refermentation method did not compromise the sensory attributes of the final wine and instead led to increased levels of desirable aroma such as isoamyl acetate as well as ethyl hexanoate (Vilela-Moura et al. 2010b). *L*. *thermotolerans* was identified as one of the yeast isolates observed to consume glucose and acetic acid at an efficiency close to that of *S*. *cerevisiae* under aerobic conditions. However, the efficiency of acid consumption was lowered under limited-aerobic conditions and the acetic acid capabilities of the yeast were not further explored (Vilela-Moura et al. 2008). Fermentation with selected strains of *L*. *thermotolerans* have been reported to result in a lower amount of acetic acid in comparison to *S*. *cerevisiae*. Similar observations were made with strains of *T*. *delbrueckii*, *H. uvarum*, *C. stellata* and *C. zemplinina* (Rantsiou et al. 2012; Gobbi et al. 2013; de Kock 2015). Wine-related yeasts such as *Z. bailii*, *T. delbrueckii*, *C. utilis* and *Dekkera anomala* were also reported to consume acetic acid (Casal and Cardoso 1996; Rodrigues et al. 2012; Vilela et al. 2015) but the ability to consume acetic acid under wine conditions were not thoroughly explored. Thus further investigations are required to determine whether non-*Saccharomyces* yeasts maintain low volatile acidity through acid consumption during the early stages of fermentation when the conditions are semi-aerobic.

2.8 Conclusions and future outlooks

Non-*Saccharomyces* yeasts are currently selected for winemaking in an attempt to improve wine complexity and/or diversify wine aromatic styles. In literature, these unconventional yeasts are reported to produce variable amounts of glycerol and some selected strains were observed to maintain low acetic acid levels during alcoholic fermentation. A variety of polyols were

detected in table as well as Botrytis affected wine and it was assumed that yeasts, bacteria and molds may be responsible for their production. Indeed non-*Saccharomyces* yeasts are capable of producing additional polyols under a variety of conditions. Since glycerol is known to impart a rounder mouthfeel and slight sweetness to wine, investigations in polyol production for wine related yeasts are not only required to unravel the biological function of these compounds but also to assess the sensory impact of these compounds in wine. As the wine industry is interested in yeasts producing novel/valuable metabolites, non-*Saccharomyces* strains already beneficial to winemaking should be screened for polyol production to encourage commercialisation. Furthermore, selected non-*Saccharomyces* yeasts have been observed to consume acetic acid (semi-aerobically) whereas others have been reported to maintain low levels of volatile acidity during alcoholic fermentation. Therefore, it would be worth investigating if low acetic acid producers are capable of acetic acid consumption in semi-aerobic conditions. Overall, non-*Saccharomyces* yeasts are important for improving wine complexity and the metabolic activities of such strains need to be investigated as to identify more compounds that may alter sensorial properties of the wine matrix.

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Chapter 3

Research results: Investigating polyol and acetic acid metabolism in wine related non-*Saccharomyces* yeasts

Chapter 3: Investigating polyol and acetic acid metabolism in wine related non-*Saccharomyces* yeasts

3.1 Introduction

Wine results from grape juice fermentation which involves the biochemical conversion of sugars into ethanol and carbon dioxide along with a large variety of metabolites. This transformation mainly relies on yeasts, particularly *Saccharomyces cerevisiae*. Hundreds of strains of this species have been selected and are now commercialised to the wine industry because of their ability to realise efficient, controllable and therefore fairly reproducible fermentations while producing sought-after aroma compounds. On the other hand, claims that the use of starter cultures has led to the standardisation of sensory, chemical and analytical profiles in wines have recently arisen (Capozzi et al. 2015). In an attempt to re-establish the greater complexity arising from spontaneous fermentations and/or address specific oenological challenges, the utilization of non-*Saccharomyces* yeasts has been suggested. Indeed, specific non-*Saccharomyces* yeasts have been observed to positively contribute to the wine chemical composition and therefore improve the sensory characteristics of wine (Bely et al. 2008; Canonico et al. 2015; Renault et al. 2015). Most non-*Saccharomyces* yeasts are however not strong fermenters and must be used in multi-starter fermentations, always comprising *S. cerevisiae.* The winemaker can thereby benefit from the metabolic features of the non-*Saccharomyces* yeasts while limiting the risk of stuck and unpredictable fermentations thanks to *S. cerevisiae* (Jolly et al. 2014; Wang et al. 2016).

The behaviour and performance of yeast species during fermentation is tightly linked to stress resistance such as that induced by the osmotic pressure in grape juice and redox imbalances as oxygen becomes limiting while ethanol accumulates (Erasmus et al. 2003; Munna et al. 2015; Noti et al. 2015). The ability of yeasts to overcome these stresses indeed determines the extent of their survival and ultimately the outcome of fermentation.

During winemaking, osmotic stress occurs primarily at the onset of fermentation as yeast cells encounter or are inoculated into grape must containing high levels of sugar. This causes a change in water movement along the cell membrane and water is lost from the cell as a consequence of the osmotic gradient and regulatory mechanisms are required to prevent water loss and cell death. In response to hypertonic environmental conditions, yeasts have developed strategies aimed at maintaining cell integrity through the synthesis and accumulation of compatible solutes (Logothetis et al. 2014).

In *S. cerevisiae*, the principal compatible solute is glycerol. In short, glycerol is synthesized in the cytosol from the glycolytic intermediate dihydroxyacetone phosphate in a two-step catalytic reaction with the enzymes glycerol-3-phosphate dehydrogenase (*GPD*) and glycerol-3-phosphatase (*GPP*) (Erasmus et al. 2003). In addition to protecting the cell from a variety of stressors, glycerol production has also been linked to a regulation of redox balance. Indeed, in

an anaerobic environment such as that occurring in fermenting grape juice, NADH accumulates and in turn induces a need for a regulatory mechanism to recycle this co-enzyme (Eglinton et al. 2002).

Glycerol also carries oenological importance in that it has a full and smooth mouthfeel and may therefore improve wine quality. The synthesis of glycerol leads to acetic acid production in *S. cerevisiae*. Furthermore, when acid concentrations are high, it has been linked to grape must containing excessive amounts of sugar (e.g. in late harvest grape juices). The synthesis of acetic acid functions to reinstate redox imbalances by using surplus $NAD(P)^+$ that would have accumulated during the synthesis of fermentation by-products such as glycerol (Soden et al. 2000; Eglinton et al. 2002; Li et al. 2010).

On the contrary, unlike glycerol which at certain concentrations is desirable in wine, acetic acid gives off a vinegary aroma detrimental to the quality of wine that is easily detected due to its low sensory threshold (Pigeau and Inglis, 2005).

Certain non-*Saccharomyces* yeasts, have been reported to react differently. For instance, *Starmerella bacillaris* was observed to produce high levels of glycerol without increasing levels of acetic acid while *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima* and others were reported to produce variable amounts of glycerol along with acetic acid depending on strains and fermentative conditions (Renault et al. 2009; Rantsiou et al. 2012; Gobbi et al. 2013a).

Furthermore, sugar alcohols such as arabitol, mannitol and erythritol in addition to glycerol have been detected in white, red, and noble rot wines. In all wines, glycerol was produced at the highest amount (4 - 12 g/l in white or red wine and 15 - 25 g/l in noble rot wine). D-arabitol was the second highest polyol detected (0.1 - 0.6 g/l in white or red wine and 2.3 g/l in noble rot wine) followed by 2,3-butanediol, sorbitol, mannitol and a few others (Margalit 2012). Although the producing strains were not isolated, it was assumed that bacteria, yeasts or fungi may be responsible. The production of polyols is dependent on the carbon source, growth conditions and yeast strain used. In brief, sugar alcohols can be produced from the reduction of a variety of sugars (erythritose, L-arabinose, mannose, xylose etc.) or from other metabolic routes (such as the pentose phosphate pathway) which branch off from the glycolytic cycle (Song and Vieille 2009; Toivari et al. 2010; Zhu et al. 2010). Indeed, yeasts such as *Saccharomyces mellis*, *Candida albicans*, *Hansenula anomala*, *Debaromyces napelensis* and *Zygosaccharomyces* sp. were observed to produce polyols from a range of substrates and environmental conditions with functions relating to osmotic, heat and oxidative stress resistance (Van Eck et al. 1989; Wong et al. 1993; Shen et al. 1999; Nozaki et al. 2003; Saha et al. 2007; Zhu et al. 2010; Kumdam et al. 2013). Furthermore, *L. thermotolerans*, *St. bacillaris* and *T. delbrueckii* were observed to produce a variety of polyols in addition to glycerol during alcoholic fermentation in synthetic must-like media. However, these compounds were only analysed at fermentation end and the enzyme assays used could only detect polyols in combination (De Kock 2015). Since glycerol is

known to impact wine quality, studies focusing on the production of other polyols such as sorbitol and arabitol (which are known for their sweetness) are also necessary as these compounds may impact wine mouthfeel. The aim of this study was to evaluate the production of sugar alcohols and acetic acid in non-*Saccharomyces* yeasts under different environmental conditions and to investigate already published methods that allow for the separation of polyols.

3.2. Materials and Methods

3.2.1. Yeast strains, fermentation media and conditions

Prior to fermentation, the yeast strains listed in Table 1 were streaked onto Yeast Peptone Dextrose Agar (Merck, Gauteng, South Africa) plates. A single colony was inoculated into 5 ml YPD and incubated for 24 h at 30°C and 1 ml or 2 ml of pre-cultures were transferred into 100 ml or 200 ml YPD respectively and incubated for 9 h - 12 h at 30°C until the cells reached mid-exponential growth phase. The yeasts were then inoculated at $1x10^7$ cells/ml into 150 or 350 ml synthetic grape juice-like medium (Mostert and Divol 2014), grape must or Yeast Nitrogen Base (YNB) containing ammonium sulphate (Difco, Le Pont-de-Claix ,France) and supplemented with 100 g/l sugars. For grape must studies, the nitrogen level was adjusted to 300 mg/l YAN with Thiazote® to make up 150 mg/l ammonium (Laffort oenologie, Bordeaux, France) and the amino acids mixture described in Table 3 to make up 150 mg/l (Bely 1990). This was to ensure that the YAN levels were similar in all grape must and grape-like synthetic medium. After inoculation, fermentations were incubated at 25°C with agitation at 120 rpm. Table 2 indicates in more details the different media used in this study. At each sampling point, 5 or 7 ml samples were harvested. Of that volume, 1 ml was used to follow growth of the yeast strains via OD_{600nm} or by plating on YPD agar and occasionally on Wallerstein Laboratory Nutrient Agar (Sigma, Missouri, USA) to verify the absence of any contaminants indicated by differential growth on the medium. The remaining 4- or 6-ml samples were centrifuged at 4193 *g* for 5 min. The supernatants were filtered through 0.22-µm filter (Starlab Scientific, Cape Town, South Africa) and 1 ml aliquots were transferred into microcentrifuge tubes. Samples were stored at -20°C before chemical analysis.

3.2.2. Chemical analyses using enzymatic kits

For the determination of glucose, fructose, glycerol and acetic acid concentrations, Enzytec™ Fluid kits (R-Biopharm, Germany) were used with an Arena 20XT analyser at the Central Analytical Facility (Stellenbosch University, Stellenbosch, South Africa). Furthermore, the concentrations of sugar alcohols were determined using enzyme assay kits from Megazyme (Bray, Ireland). However, the kits used did not allow to distinguish between individual polyols and results were therefore given in a combination of two polyols. In one assay, D-mannitol was detected together with L-arabitol and in another D-sorbitol was determined with D-xylitol.

Table 1: Yeast strains used in this study

Species	Strain name and Culture Collection	Region/Supplier
Saccharomyces cerevisiae	Lalvin EC1118	Lallemand Inc., Montreal, Canada
Lachancea thermotolerans	IWBT[1] Y1220	Isolated from grape must in South Africa
Torulaspora delbrueckii	IWBT[1] Y930	Isolated from grape must in South Africa
Torulaspora delbrueckii	Biodiva™	Lallemand Inc., Montreal, Canada
Torulaspora delbrueckii	CRBO[2] L0544	Isolated from grape must in France
Starmerella bacillaris	IWBT Y1283	Isolated from grape must in South Africa

[1]: Institute for Wine Biotechnology, Stellenbosch University, South Africa; [2]: Centre de Ressources Biologiques Œnologiques, Université de Bordeaux, France

3.2.3 Separation of polyols by chromatography

Thin Layer Chromatography (TLC) was used in an attempt to overcome the limitation of enzyme assays that detect polyols in combination. The protocol used was for the separation of polyols from their corresponding aldoses and modified for the detection of polyols in wine. In summary, acetonitrile-ethyl acetate-1-propanol-water (85/20/20/15 v/v/v/v) was used as a mobile phase and an Alugram Xtra K6 plate as the stationary phase (Macherey Nagel, Germany). Polyols were visualized through an alkaline silver nitrate-sodium thiosulphate dipping system (Han and Robyt 1998). As TLC is a qualitative means of analysis and also proved not to be sensitive enough for the separation of polyols, Gas Chromatography-Mass Spectrophotometry was used to separate and analyse polyols derivatized with TMS-HMDS-pyridine as described by York et al. 1985 with a few modifications at the Central Analytical Facility (Stellenbosch University, Stellenbosch, South Africa). Briefly, the samples were converted to their methoxy form using 1 M methanolic HCl at 80°C for 16 h. After silylation, the derivatives were separated and analysed in a gas chromatograph, Agilent 6890 N (Agilent, Palo Alto, CA) coupled to a Agilent 5975 MS mass spectrometer detector, using a polar (95% dimethylpolysiloxane) ZB-Semivolatiles Guardian (30 m, 0.25 mm ID, 0.25 µm film thickness) part number 7HG-G027-11GC column. The oven temperature program was maintained at 80°C for 1 min and finally ramped at 7°C/min to 300°C and then held for 2 min. The carrier gas was helium with a flow rate of 1 ml/min and the injector temperature was maintained at 280°C in a splitless mode. The mass spectral data was recorded on a MSD operated in full scan mode (40-650 *m/z*) with both the ion source and quadrupole temperatures maintained at 240°C and 150°C respectively. The

transfer line temperature was maintained at 280°C. Samples were quantified and integrated according to retention times using polyol mixtures using methods described in previous reports (York et al. 1985; Gao et al. 2015).

Table 2: Media and yeast strains used for alcoholic fermentation

FERMENTATION MEDIA	SUGAR CONCENTRATION (1:1 GLUCOSE:FRUCTOSE)	YEAST STRAINS USED
INFLUENCE OF INITIAL SUGAR CONCENTRATION		
Synthetic grape juice-like medium	230 g/l	*L. thermotolerans*, *T. delbrueckii* CRBO L0544, *St. bacillaris* and *S. cerevisiae*
Synthetic grape juice-like medium	120 g/l	*T. delbrueckii* CRBO L0544, *T. debrueckii* IWBT Y930, *T. delbrueckii Biodiva* and *S. cerevisiae*
Synthetic grape juice-like medium	50 g/l	*T. delbrueckii* CRBO L0544 and *S. cerevisiae*
Chenin blanc grape must	230 g/l	*T. delbrueckii* CRBO L0544 and *S. cerevisiae* pure. Sequential (*S. cerevisiae* 48h later)
Chenin blanc grape must	120 g/l	*T. delbr*ueckii CRBO L0544 and *S. cerevisiae*
INFLUENCE OF INITIAL NaCl CONCENTRATION		
Synthetic grape juice-like medium	100 g/l	*T. delbr*ueckii CRBO L0544 and *S. cerevisiae*
Synthetic grape juice-like medium with 0.05 M NaCl, 0.1M NaCl or 0.5 M NaCl		
INFLUENCE OF THE PRESENCE OF ACETIC ACID OR ETHANOL		
Synthetic grape juice-like medium	100 g/l	*T. delbr*ueckii CRBO L0544 and *S. cerevisiae*
Synthetic grape juice-like medium supplemented with 0.12 g/l acetic acid or 4% ethanol		
INFLUENCE OF THE AVAILABILITY OF NUTRIENTS OTHER THAN SUGARS		
YNB	100 g/l	*T. delbr*ueckii CRBO L0544 and *S. cerevisiae*
YNB with MS300 amino acids (150 mg YAN)		
YNB with lipids (100 mg Ergosterol and 5 ml Tween 80)		

Table 3: Amino acid composition of synthetic grape juice-like medium (Bely et al. 1990)

Amino acid	g/l	Amino acid	g/l
Tyrosine	1.4	Alanine	11.1
Tryptophane	13.7	Valine	3.4
Isoleucine	2.5	Methionine	2.4
Aspartic acid	3.4	Phenylalanine	2.9
Glutamic acid	9.2	Serine	6a
Arginine	28.6	Histidine	2.5
Leucine	3.7	Lysine	1.3
Threonine	5.8	Cysteine	1
Glycine	1.4	Proline	46.8
Glutamine	38.6	Ammomium sulphate	0.46

3.3. Results

3.3.1. Optimization of techniques used for the separation of polyols

The aim of this study was to investigate polyol production in non-*Saccharomyces* yeasts and in any study, it is important to ensure that the methods used for the analyses of compounds of interest are accurate that 1) the study is reproducible and 2) correct conclusions are drawn. Although the assay used for the detection of glycerol was specific for this compound, the enzyme assays used for detection and quantification of the other polyols were limited to detecting polyols in combination (D- mannitol with L-arabitol and D-sorbitol with D-xylitol). As a result, a variety of methods were employed in an attempt to separate these compounds.

3.3.1.1. TLC

A TLC protocol based on the separation of sugars from their corresponding sugar alcohols (Han and Robyt 1998) was used. The protocol was also selected because it did not detect glycerol which was found at much higher levels than the other polyols and would have made the detection of these compounds difficult. After several attempts and modifications of the original protocol (Table 4), this method proved partially successful for the detection of individual compounds but only when concentrations were above 500 ng (Figure 1a and S1). However, the method was unsuccessful when a polyol mixture was spotted. Indeed, D-Mannitol and D-sorbitol could not resolve from one another since they migrated too closely from one another and the same scenario occured with D-xylitol and D-arabitol (Figure 1a, lane PM). Furthermore, when

Table 4: Optimization of a Thin Layer Chromatography protocol for the separation of polyols

Thin Layer Chromatography				
Attempts in chronological order and samples spotted	**Mobile phase**	**Dipping system**	**Destaining and removal of background**	**Results**
	Solvent	Reagents	Reagent	
1. Spotted 1 mg polyol standard (Han and Robyt 1998)	Acetonitrile-ethyl acetate-propanol-1-water (85:20:20:15 v/v/v/v) ➢ Irrigated plate twice ➢ Dry in between ascents	➢ Alkaline silver nitrate ➢ Dipped plate for 5 min and dried in air ➢ Alkaline methanol ➢ Dipped plate for 30 min- brown spots were supposed to appear for carbohydrates (polyols) and dried in air	Sodium thiosulphate (1.5M) ➢ Dipped plate to remove background for 5 min ➢ Wash for 1 min under running water	• Smears were observed after dipping in alkaline methanol • Hypothesized that the staining system may not be sensitive enough or that the concentration of standards used was too high • Plate degraded after using sodium thiosulphate-concentration may be too high
2. Spotted various concentrations (500 ng-5 µg) of mannitol and sorbitol to test sensitivity of staining system	-	➢ Alkaline silver nitrate ➢ Dipped plate for 30-90 seconds with shaking ➢ Alkaline methanol ➢ Dipped plate for 30-90 seconds and black spots were immediately observed	-	• Since the staining method was observed not to be the problem (see Figure S1a), it was decided to decrease the concentration of standards
3. Spotted 10µg polyol standards individually and in a mixture. Also spotted *T. delbrueckii* and *S. cerevisiae* samples	Performed as indicated in attempt 1	Performed as indicated in attempt 2	Sodium thiosulphate (0.5M) ➢ Dipped plate to remove background for 1 min	• Compounds resolved well individually but did not separate well in the polyol mixture-change the ratios of solvents in mobile phase • Mannitol and sorbitol resolved together and same was observed for xylitol and mannitol • Too much background in the fermentation samples • Plate did not degrade after removal of background-sodium thiosulphate concentration was good for destaining
4. Spotted polyol mixture as in attempt 2	Acetonitrile-ethyl acetate-propanol-1-water ➢ 55:35:35:15 v/v/v/v ➢ 50:55:20:15 v/v/v/v	Performed as indicated in attempt 2	Performed as indicated in attempt 2	• Mannitol and sorbitol resolved together. Similar observations were made for arabitol and xylitol • The separation of polyols is dependent on adsorption which is in turn dependent on polarity difference-the compounds that resolve together may have similar affinities to the mobile phase (regardless of solvent ratios used in this study) and stationary phase making separation difficult

the detection of these polyols was attempted in the supernatants of *T. delbrueckii* and *S. cerevisiae* fermentations, a dark background, resulting in smears instead of clear spots prevented the accurate visualization of these compounds (Figure 1a, lanes TD and SC, respectively). D-arabitol was tested in this experiment instead of L-arabitol that is detected in the enzyme assay, because the latter compound cannot been produced by the yeasts in grape juice or synthetic grape juice-like medium as it requires L-arabinose as substrate and this monosaccharide is not present in the synthetic media used in this study. On the other hand, D-arabitol can be produced from the metabolism of glucose and fructose.

Table 5: Optimization of a Gas Chromatography-Mass Spectrophotometry protocol for the separation of polyols

Gas Chromatography-Mass Spectrophotometry				
Attempts in chronological order and samples analysed	**Drying and Sugar extraction**	**Derivitazation**	**Reconstitution of samples**	**Results**
1. Used single level of standards	➢ Dried samples (100 µl) under nitrogen flow at 60°C ➢ Added 500 µl methanolic HCL solution (1:2) ➢ Incubated samples for 16h at 80°C	➢ Dried samples and washed with dried methanol twice ➢ Added 150 µl TMS –pyridine-HMDS ➢ Incubated at 80°C for 20 min ➢ Dried samples under nitrogen at 40°C	➢ Reconstituted samples in 1500 µl cyclohexane and use 1000 µl for analyses	• Compounds resolved well
2. Used 100 µl *T. delbrueckii* and *S. cerevisiae* samples from fermentation containing 230 g/l sugars	➢ Performed as indicated in 1	➢ Performed as indicated in 1	➢ Performed as indicated in 1	• Polyol extraction was unsuccessful for *T. delbrueckii* samples due to the caramelization of residual sugars from fermentation
3. Used 100 µl *T. delbrueckii* and *S. cerevisiae* samples from fermentation with 100 or 120 g/l sugars. Included standard curve (10, 20, 50, 100, and 250 ppm) with pentaerythritol (10 ppm) as internal standard.	➢ Performed as indicated in 1	➢ Added 150 µl TMS –pyridine-HMDS ➢ Incubate at 80°C for 20 min	➢ Spin down debris and use supernatant for analyses	• Standard curve was linear and there was no interference with the internal standard. Fermentation samples were successfully quantified and integrated.

3.3.1.2. GC-MS

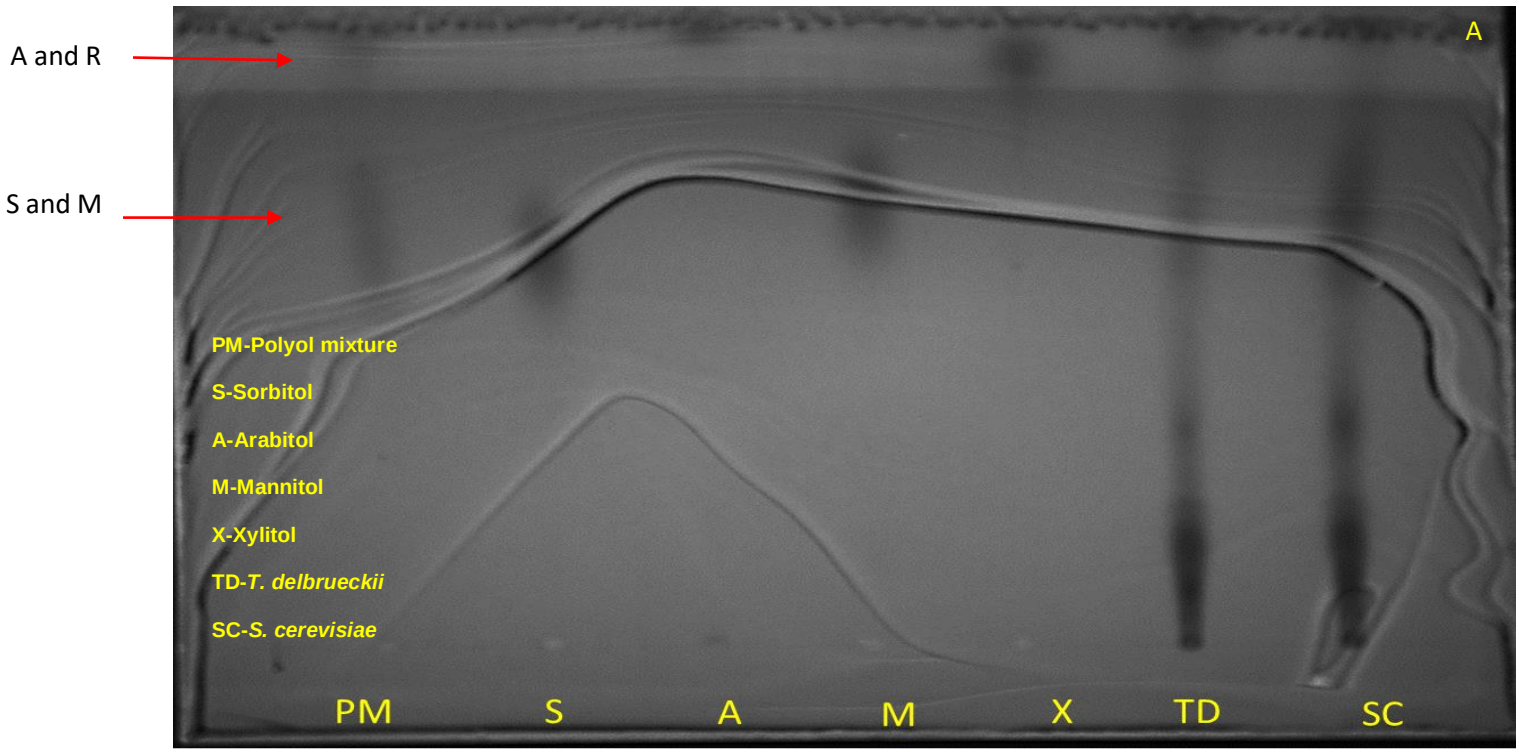
Since the TLC method proved not to be sensitive enough for the separation of polyols and was already disadvantageous by only being qualitative, another method was selected and optimized.

A GC-MS protocol based on the separation of sugars within cell wall components with some modifications was used for the separation of polyols (York et al. 1985; Gao et al. 2015). The different optimization steps are summarized in Table 5. This protocol was observed to be limited in only detecting polyols in dry fermentation samples because those containing residual sugars caramelized during the sugar extraction stage. Nonetheless, after some optimization (Table 5), this method proved successful for the separation of polyols (Figure 1b) and was used for the analysis of dry fermentation samples throughout this study. The method was validated as shown in Table 6. Despite the limitations highlighted above, the enzymatic kits were nevertheless used for the monitoring of polyol production during fermentations as sugars did not interfere with the results, unlike with the GC-MS method.

Table 6: Validation parameters for separation of polyols using Gas Chromatography-Mass Spectrophotometry

Polyol Standards	R^2	LOQ (mg/l)	LOD (mg/l)
Ribitol	0.9985	18.9105	5.6732
D-Arabitol	0.9878	125.6787	37.7036
D-Xylitol	0.9877	126.3230	37.8969
D-Mannitol	0.9822	152.6715	45.8014
D-Sorbitol	0.9856	136.7934	41.0380

Keys: R squared (R^2); Limit of Quantification (LOQ); Limit of Detection (LOD)



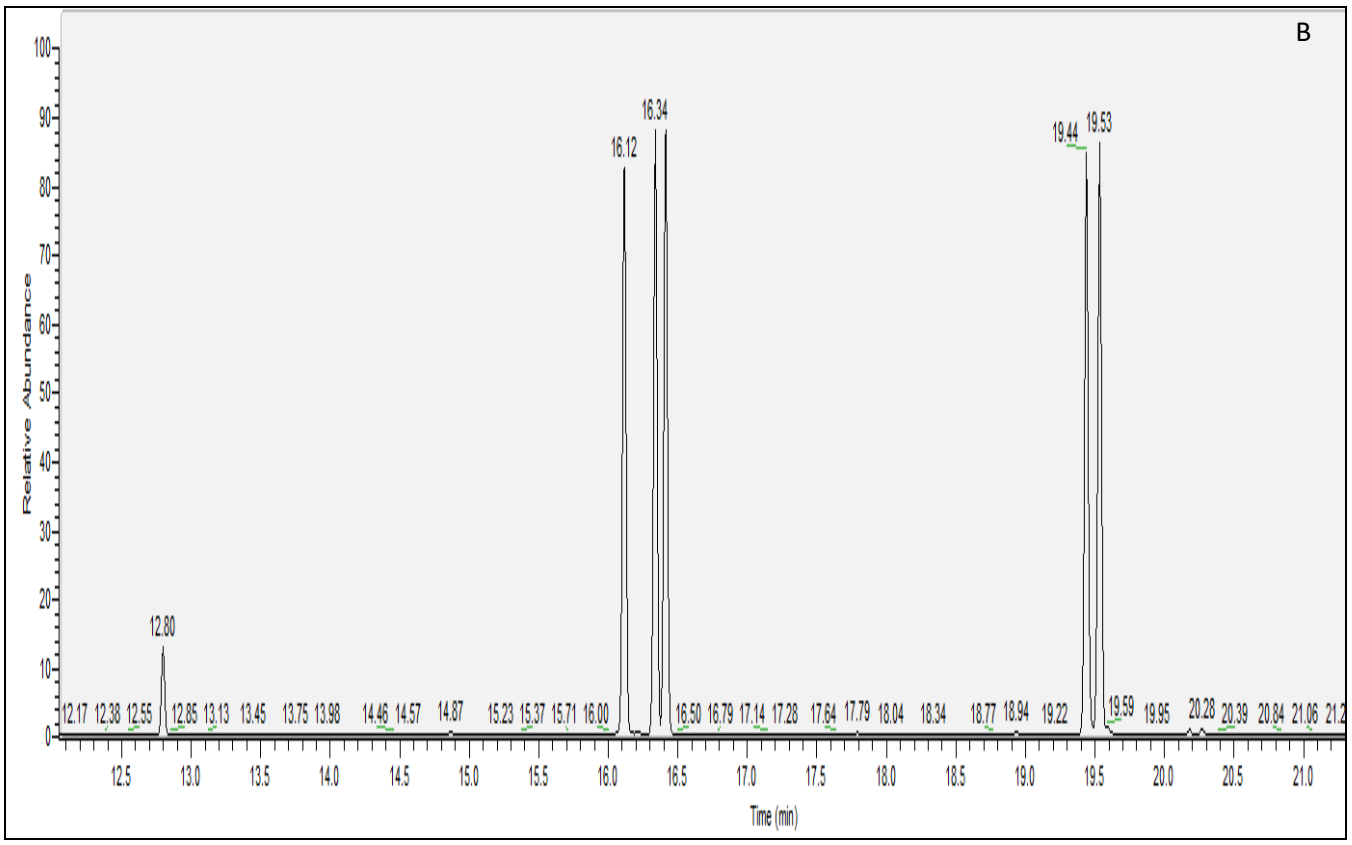


Figure 1: Separation of polyols: A) Resolution of polyols on a K6 sheet using TLC. PM-polyol mix; S-sorbitol; A-arabitol; M-mannitol; X-xylitol; TD-*Torulaspora delbrueckii*; SC-*Saccharomyces cerevisiae,* B) Separation of polyol standards using GC-MS: Pentaerythritol (12:80), Ribitol (16:12), D-xylitol (16:34), D-arabitol (16:37); D-mannitol (19:44) and D-sorbitol (19:53).

3.3.2. Screening of non-*Saccharomyces* yeasts and strains for polyol production

3.3.2.1 Population dynamics and fermentation rate

Non-*Saccharomyces* yeasts were screened for sugar alcohol production during alcoholic fermentation. Pure cultures of *St. bacillaris*, *L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* were inoculated at a concentration of $1x10^7$ cfu/ml into 350 ml synthetic grape juice-like medium containing 230 g/l sugars. Growth determined through cell counts and fermentation progress monitored through the amount of CO_2 released are shown in Figure 2. *St. bacillaris* and *T. delbrueckii* persisted throughout fermentation with a population close to that observed for *S. cerevisiae* by day 11. *L. thermotolerans* maintained the lowest population throughout fermentation and its population declined by day 6. As expected, *S. cerevisiae* fermented efficiently and consumed all sugars by day 5. Although *T. delbrueckii* consumed all glucose by day 9, the fermentation was stuck around the same time and a residual fructose concentration of 18.74 g/l was observed. Fermentation was stuck on day 5 in *L. thermotolerans* resulting in a residual sugar concentration of 42.9 g/l. For *St. bacillaris*, the low amount of CO_2 released in Figure 2b is further accounted for by its strong fructophillic nature. Indeed, Figure 3 shows that *St. bacillaris* depleted fructose by day 4 while 68.5 g/l residual glucose was observed.

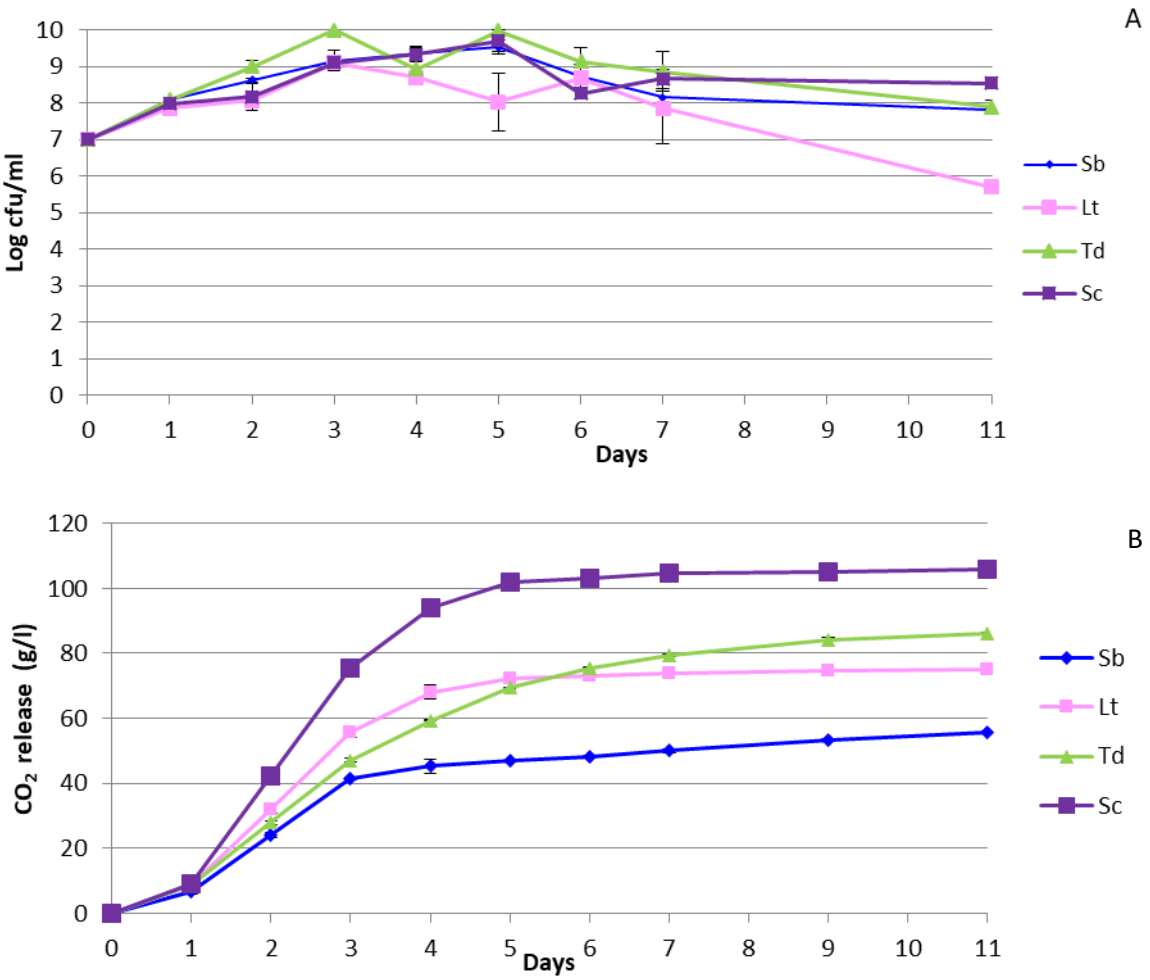


Figure 2: Growth and fermentation of yeast strains in high sugar synthetic must: A) Yeast population dynamics; B) Fermentation kinetics. Sb-*Starmerella bacillaris*, Lt-*Lachancea thermotolerans*, Td-*Torulaspora delbrueckii*, Sc-*Saccharomyces cerevisiae*.

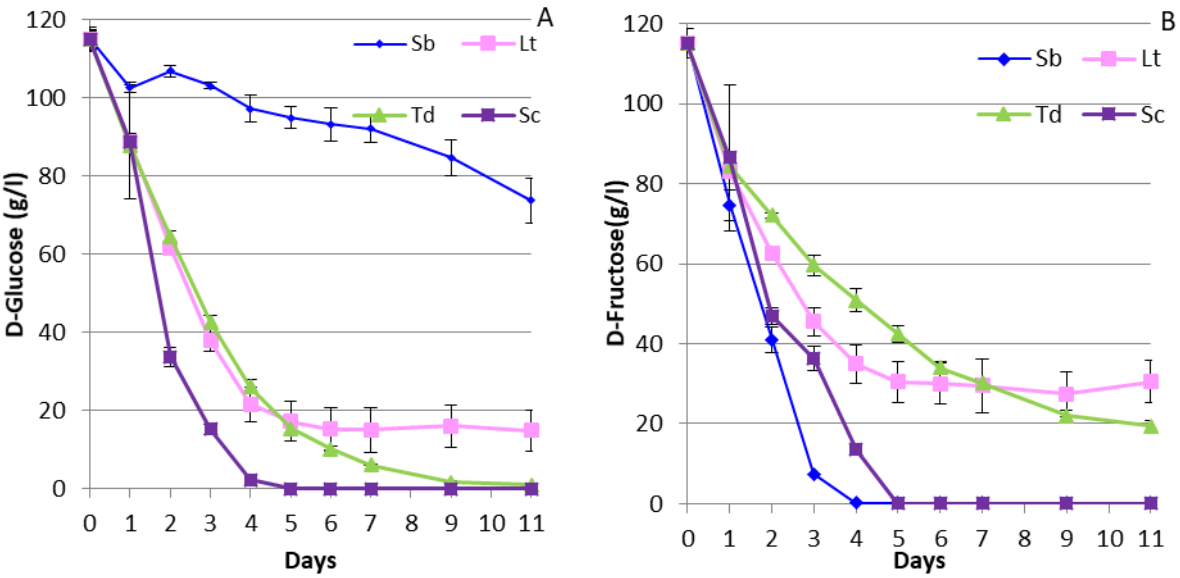


Figure 3: Sugar consumption in high sugar synthetic must: A) Glucose; B) Fructose. Sb-*Starmerella bacillaris*, Lt-*Lachancea thermotolerans*, Td-*Torulaspora delbrueckii*, Sc-*Saccharomyces cerevisiae.*

3.3.2.2 Production of sugar alcohols and acetic acid

Glycerol and acetic acid concentrations were monitored throughout fermentation (Figure 4). For all strains, a sharp increase in the production of acetic acid and glycerol was observed from days 0 to 3 followed by a plateau phase. Overall, *St*. *bacillaris* was the highest glycerol producer (ca. 8 g/l) while maintaining the lowest amount of acetic acid (ca. 0.25 g/l). In contrast, *S. cerevisiae* produced low amounts of glycerol (ca. 4 g/l) and the highest amount of acetic acid (ca. 1 g/l). *T. delbrueckii* produced similar amounts of glycerol and lower levels of acetic acid concentrations in comparison to *S. cerevisiae*. Interestingly, for *T. delbrueckii*, an increase in acetic acid levels was observed from day 6 to 7 which corresponded to the time when the population started to decline. The amount of glycerol produced by *L. thermotolerans* was similar to *S. cerevisiae* and *T. delbrueckii* while acetic acid levels were similar to *St. bacillaris*.

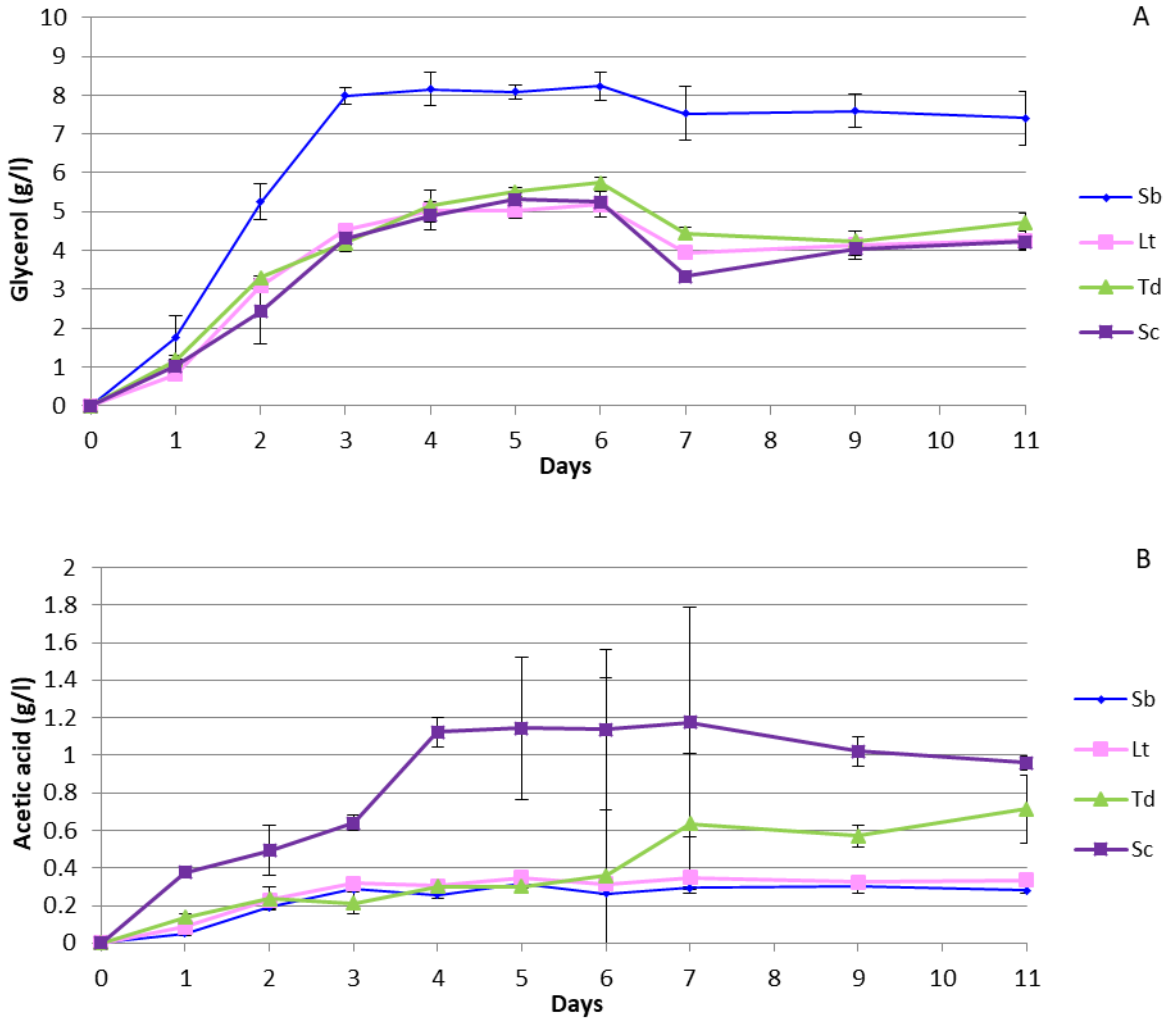


Figure 4: Primary metabolite production in high sugar synthetic must: A) Glycerol; B) Acetic acid. Sb-*Starmerella bacillaris*, Lt-*Lachancea thermotolerans*, Td-*Torulaspora delbrueckii*, Sc-*Saccharomyces cerevisiae*

Figure 5 shows the production of sorbitol/xylitol and mannitol/arabitol at selected time points throughout fermentation as established through enzymatic quantification. Although the order of production between the strains was similar for both polyol combinations, the trends observed between the two assays varied significantly. Overall, *T. delbrueckii* was the highest producer (ca. 400 mg/l mannitol/arabitol and ca. 650 mg/l sorbitol/xylitol), followed by *L. thermotolerans* (ca. 110 mg/l mannitol/arabitol and ca. 100 mg/l sorbitol/xylitol), *St. bacillaris* and *S. cerevisiae*. A steady increase in sorbitol/xylitol was observed between day 3 and day 7 followed by a plateau phase in *T. delbrueckii* while *L. thermotolerans* constantly maintained lower levels. On the other hand, the mannitol/arabitol concentration increased in the same time period as sorbitol/xylitol but decreased sharply after day 7 for all yeast strains. Indeed, *L. thermotolerans* and *T. delbrueckii* were able to use mannitol and sorbitol when provided as sole carbon sources (Supplementary data, Table S1). *St. bacillaris* and *S. cerevisiae* produced the lowest amounts of sugar alcohols (lower than 100 mg/l). These yeasts were also unable to utilize mannitol and sorbitol (Table S1).

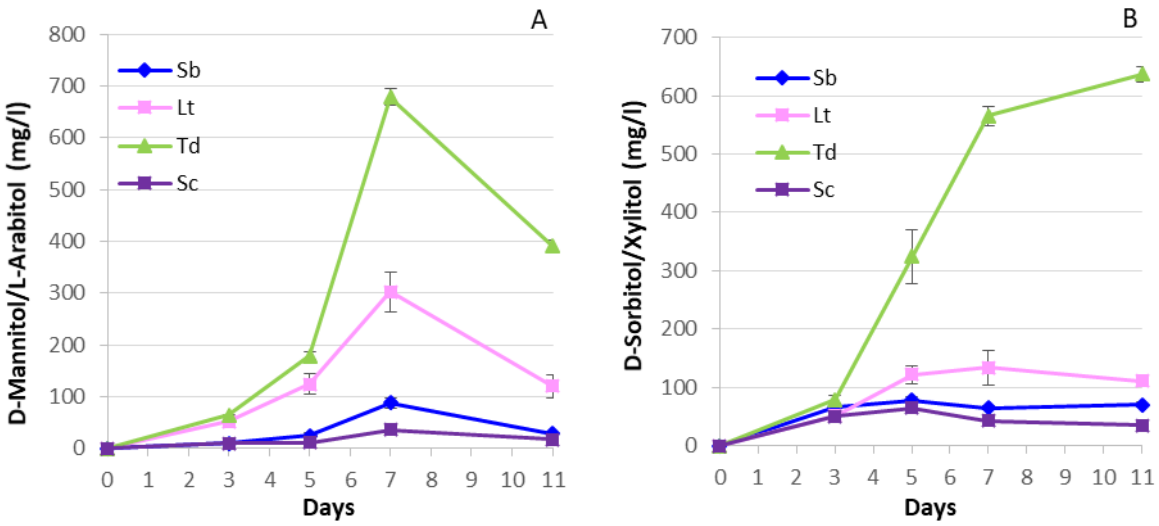


Figure 5: Sugar alcohol production during fermentation in high sugar synthetic must: A) D-Mannitol/L-Arabitol; B) D-Sorbitol/Xylitol. Sb-*Starmerella bacillaris*, Lt-*Lachancea thermotolerans*, Td-*Torulaspora delbrueckii*, Sc-*Saccharomyces cerevisiae*

3.3.2.3 Screening *T. delbrueckii* strains for polyol production

Since *T. delbrueckii* was observed to produce significant amounts of polyols during alcoholic fermentation, this yeast was selected for further studies. The impact of strain variability on polyol production in *T. delbrueckii* was investigated. Fermentations were performed in synthetic media containing 120 g/l sugars in order to ensure that all strains fermented to dryness and that final polyol concentrations could be determined by GC-MS without sugar interference. The fermentations were also repeated with 50 g/L initial sugars (with strain *T. delbrueckii* L0544

only) in order to investigate the impact of initial sugar concentration on the production of polyols. As observed in Figure 6, *T. delbrueckii* IWBT Y930 fermented the slowest whereas the other strains fermented more efficiently with *S. cerevisiae* performing the fastest. As a consequence of its slow fermentation rate, *T. delbrueckii* Y930 consumed all sugars by day 4 while the other strains completed the fermentation one day earlier.

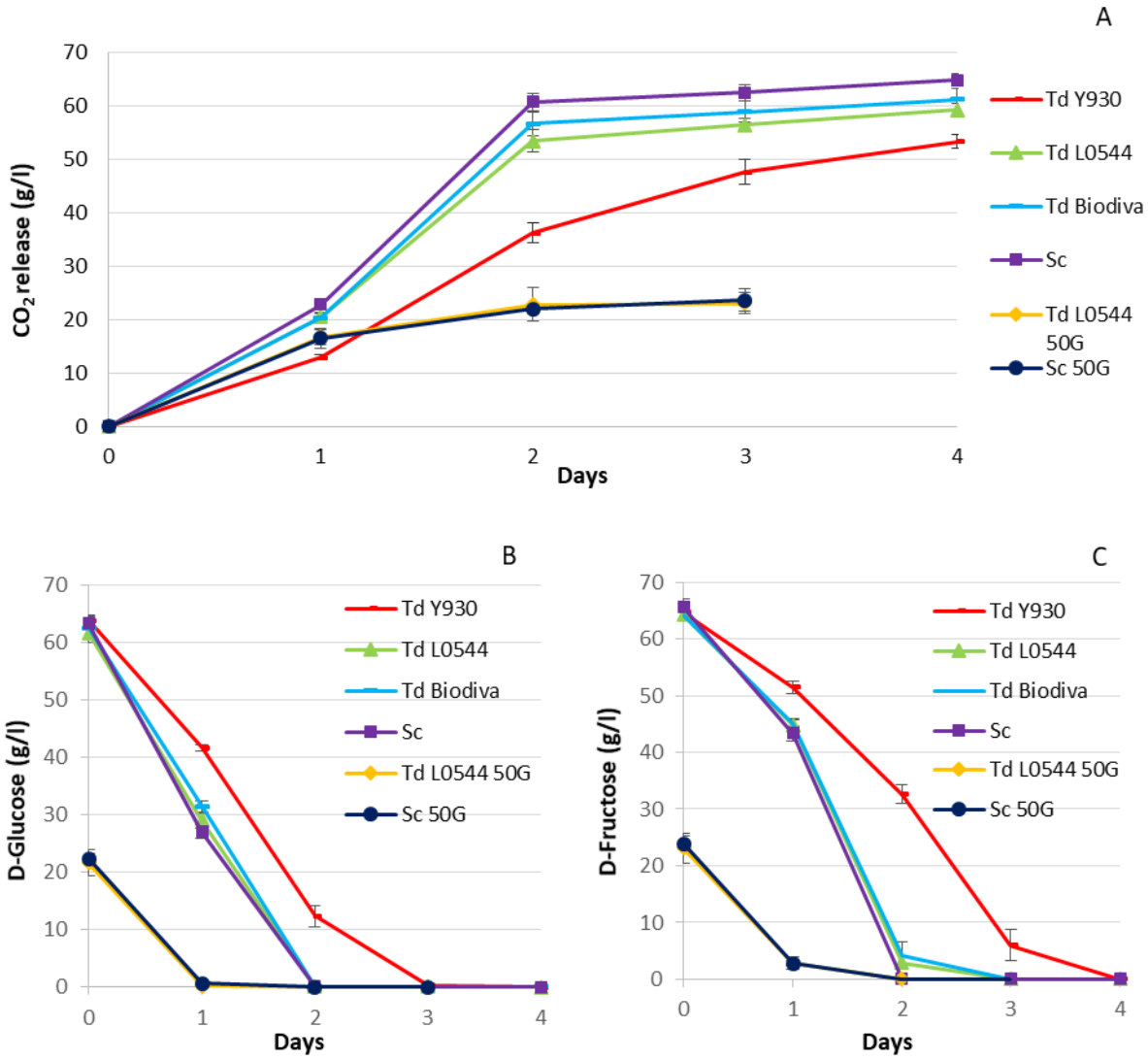


Figure 6: Fermentation kinetics in low sugar synthetic media: A) Fermentation rate; B) Glucose consumption; C) Fructose consumption. Td- *T. delbrueckii* in 120 g/l synthetic must, Sc-*S. cerevisiae* in 120 g/l synthetic must, Td L0544 50G- *T. delbrueckii* CRBO L0544 in 50 g/l synthetic must, Sc 50G-*S. cerevisiae* in 50 g/l synthetic must.

In the medium containing 50 g/l sugars, fermentations with *T. delbrueckii* L0544 and *S. cerevisiae* (Td L0544 50G and Sc 50G) were completed by day 3 and around 3.6 g/l glycerol was detected as indicated in Figure 7a. *T.delbrueckii* Y930 and *S. cerevisiae* produced about 5 g/l glycerol whereas around 6 g/l glycerol was observed in the fermentations conducted by *T. delbrueckii* Biodiva and CRBO L0544 by the end of fermentation. With the exception of *S. cerevisiae* in 50 g/l sugar must, glycerol was synthesized during the first 2 days of fermentation

where after it levelled off in all strains. Furthermore, the final acetic acid levels were lower than 0.14 g/l in all fermentations (data not shown). Similarly to the previous fermentation, sorbitol/xylitol was detected at higher amounts than mannitol/arabitol, as shown in Figure 7b and 7c. Although *T. delbrueckii* IWBT Y930 fermented the slowest, the highest levels of polyols were detected in both enzyme assays (around 150 mg/l mannitol/arabitol and 280 mg/l sorbitol/xylitol). *T. delbrueckii* CRBO L0544 produced the second highest levels of polyols (around 130 mg/l mannitol/arabitol and 250 mg/l sorbitol/xylitol) followed by Biodiva (around 120 mg/l mannitol/arabitol and 200 mg/l sorbitol/xylitol). Overall, very little strain variability was identified in terms of polyol production for *T. delbrueckii*. The lowest levels of mannitol/arabitol and sorbitol/xylitol were detected in *S. cerevisiae* regardless of sugar concentration.

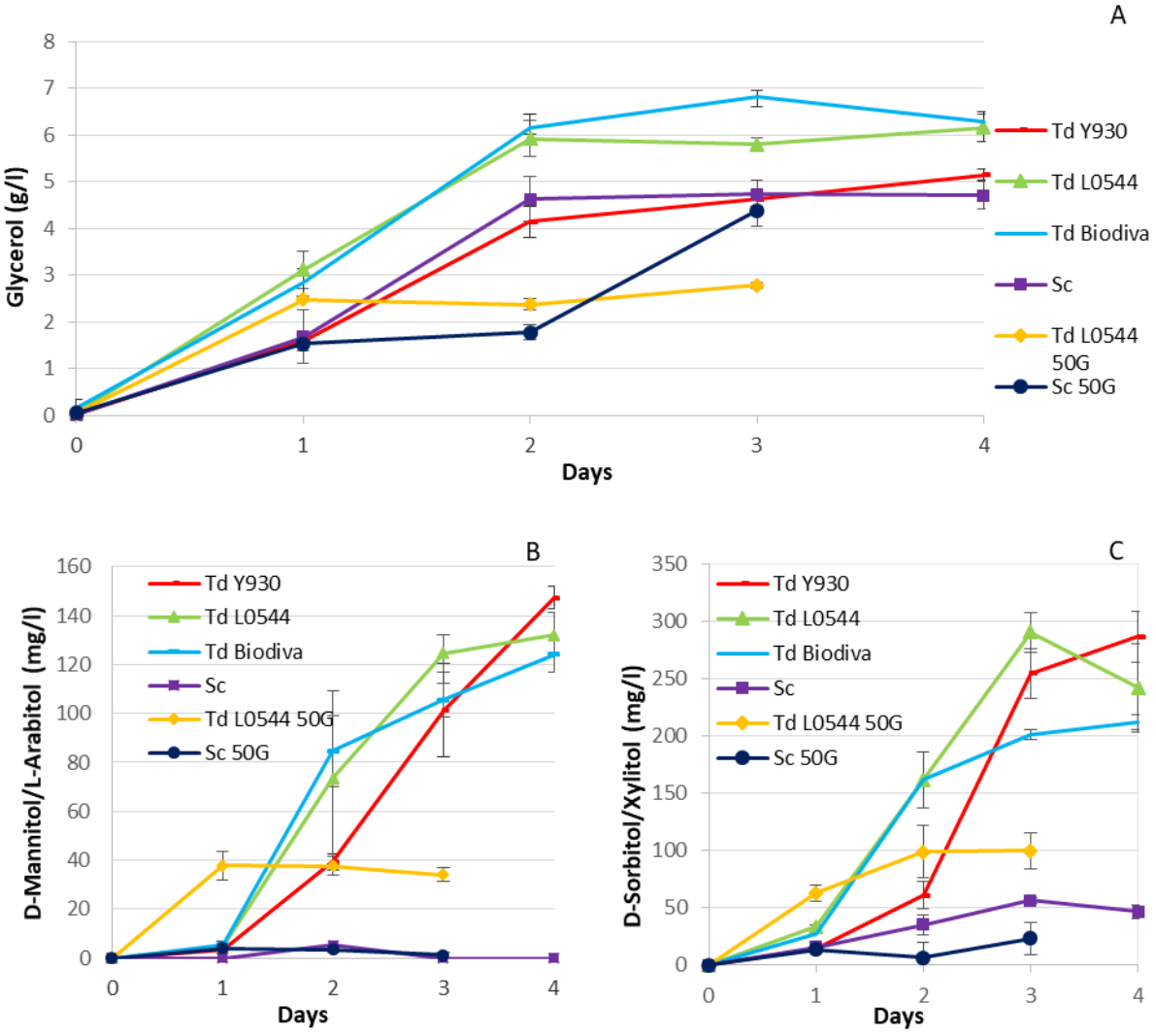


Figure 7: Sugar alcohol production in low sugar synthetic media: A) Glycerol B) D-Mannitol/L-Arabitol; C) D-Sorbitol/xylitol. Td- *T. delbrueckii* in 120 g/l synthetic must, Sc-*S. cerevisiae* in 120 g/l synthetic must, Td L0544 50G- *T. delbrueckii* CRBO L0544 in 50 g/l synthetic must, Sc 50G-*S. cerevisiae* in 50 g/l synthetic must.

At the end of fermentation, the concentrations of individual polyols were determined by GC-MS as indicated in Figure 8. All *T. delbrueckii* strains were able to produce D-mannitol, D-sorbitol

and D-arabitol with little variability. However, in this section and throughout the study, no xylitol and ribitol were detected. The amount of sugars consumed was observed to impact the levels of polyols detected. Indeed, it was observed that the higher the amount of sugars consumed, the higher levels of polyols produced in *T. delbrueckii*.

In one kit, D-mannitol and L-arabitol were analyzed together, and as indicated in Figure 8, all strains were capable of producing about 150 mg/l D-mannitol (which was similar to the amounts detected with the enzyme assays). Furthermore, L-arabitol (which results from the reduction of L-arabinose) was not used as the substrate for the synthesis of this compound was not added to synthetic must. However, D-arabitol (which derives from glucose and fructose via the pentose phosphate pathway) was detected by GC-MS in the highest amounts (ca. 450 mg/l). D-sorbitol was also detected in all strains but the amounts detected were slightly less than what was observed in the enzyme assays (around 200 mg/l D-sorbitol versus 280 mg/l sorbitol/xylitol).

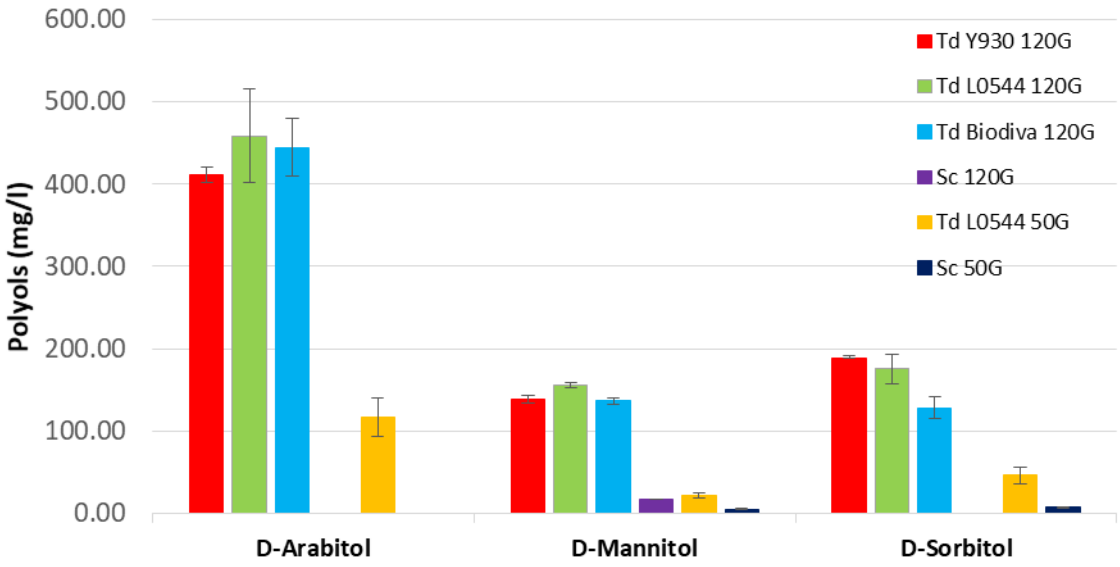


Figure 8: Polyol production at fermentation end in low sugar synthetic must. Td- *T. delbrueckii* in 120 g/l synthetic must, Sc-*S. cerevisiae* in 120 g/l synthetic must, Td L0544 50G- *T. delbrueckii* CRBO L0544 in 50 g/l synthetic must, Sc 50G-*S. cerevisiae* in 50 g/l synthetic must.

3.3.2 The synthesis of polyols in Chenin blanc must

3.3.3.1. High sugar must

Fermentations were repeated in grape must containing 230 g/L sugars. In this scenario, closer to an industrial fermentation, mixed culture fermentations were carried out in parallel to the pure culture fermentations and polyol production was monitored. In the mixed fermentation, *T. delbrueckii* was inoculated on day 0 followed by *S. cerevisiae* 48 h later, similarly to what would be carried out in industry. As expected, *S. cerevisiae* was observed to ferment efficiently. The yeast depleted glucose within 7 days and fructose within 9 days whereas sugars were only fully

consumed on day 11 in the sequential fermentation (Figure 9b and 9c) and a stuck fermentation was observed for *T. delbrueckii* with a residual sugar concentration of 22 g/l. In Figure 10a, the highest level of glycerol (7.9 g/l) was observed for *S. cerevisiae* whereas *T. delbrueckii* produced the lowest amount of glycerol (5.6 g/l). In the mixed fermentation, 6.5 g/l glycerol was produced. For all fermentations, glycerol was mostly produced within the first 3 days where after it plateaued. Similarly to previous fermentations, the amount of mannitol/arabitol produced was lower than that of sorbitol/xylitol and *T. delbrueckii* produced the highest levels of these polyols. The amount of mannitol/arabitol produced was higher in the mixed fermentation between days 3-9 in comparison to *T. delbrueckii*. Nonetheless, *T. delbrueckii* produced the highest level of mannitol/arabitol by fermentation end. By day 11, about 2500 mg/l sorbitol/xylitol was detected in *T. delbrueckii* and intermediate concentrations were observed in the mixed fermentation (1500 mg/l). *S. cerevisiae* produced the lowest amounts of sorbitol/xylitol and mannitol/arabitol throughout fermentation.

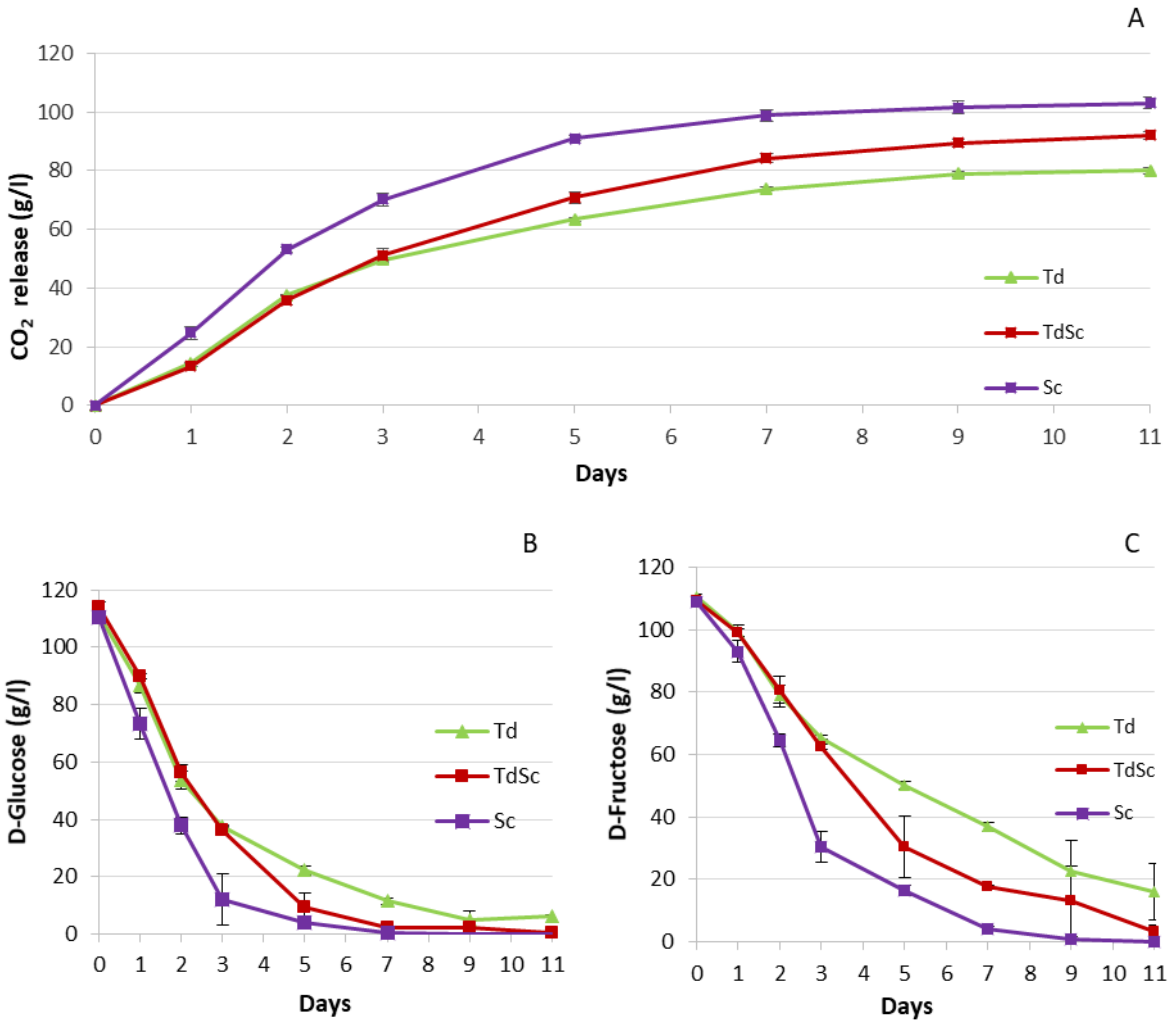


Figure 9: Fermentation kinetics in high sugar grape must: A) Fermentation rate; B) Glucose consumption and C) Fructose consumption. Td- *T. delbrueckii*, Sc-*S. cerevisiae,* TdSc- *T. delbrueckii* + *S. cerevisiae.*

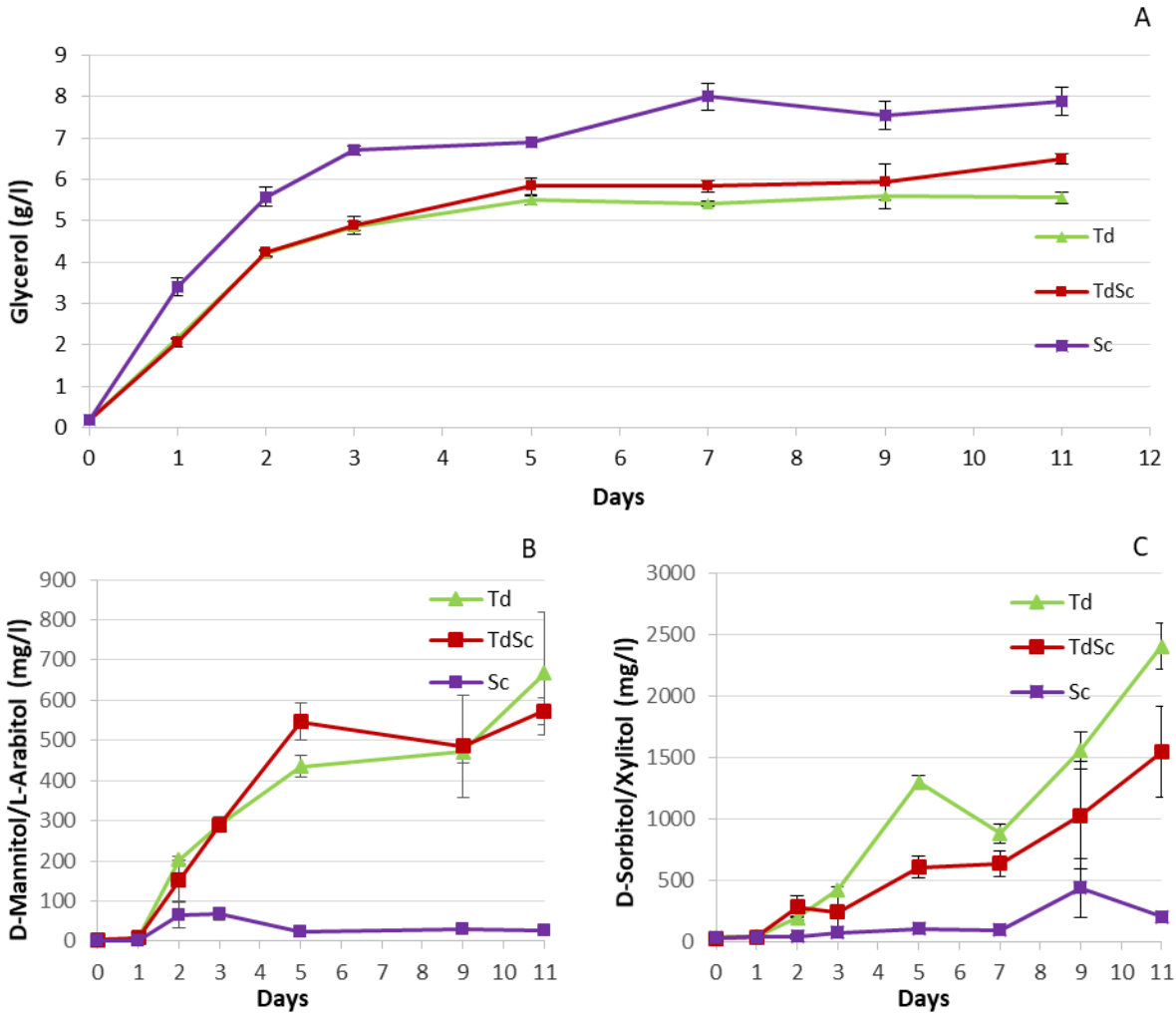


Figure 10: Sugar alcohol production in high sugar grape must: A) Glycerol; B) D-Mannitol/L-Arabitol; C) D-Sorbitol/xylitol. Td- *T. delbrueckii*, Sc-*S. cerevisiae.*

3.3.3.2. Low sugar must

In this section, fermentations were performed in diluted grape juice containing 120 g/l sugars (initially containing 260 g/l sugars) to confirm the impact of sugar concentration on polyol production. Under these conditions, fermentation was complete by day 3 and day 5 in *S. cerevisiae* and *T. delbrueckii*, respectively (Figure 11). These yeasts produced glycerol from onset of fermentation and the levels plateaued towards the end. Furthermore, Figure 12a shows *T. delbrueckii* produced slightly more glycerol (ca 5.2 g/l) than *S. cerevisiae* (ca. 4.8 g/l). Similarly to synthetic grape juice-like medium, *T. delbrueckii* produced high amounts of D-mannitol/L-arabitol and D-sorbitol/xylitol. For this yeast, increasing levels of polyols were observed from day 1-3 with production levelling off with at a final concentration of 800 mg/l for sorbitol/xylitol and 300 mg/l for mannitol/arabitol (Figure 12b and 12c). In *S. cerevisiae*, low levels of these sugar alcohols were detected throughout fermentation with no significant pattern of production.

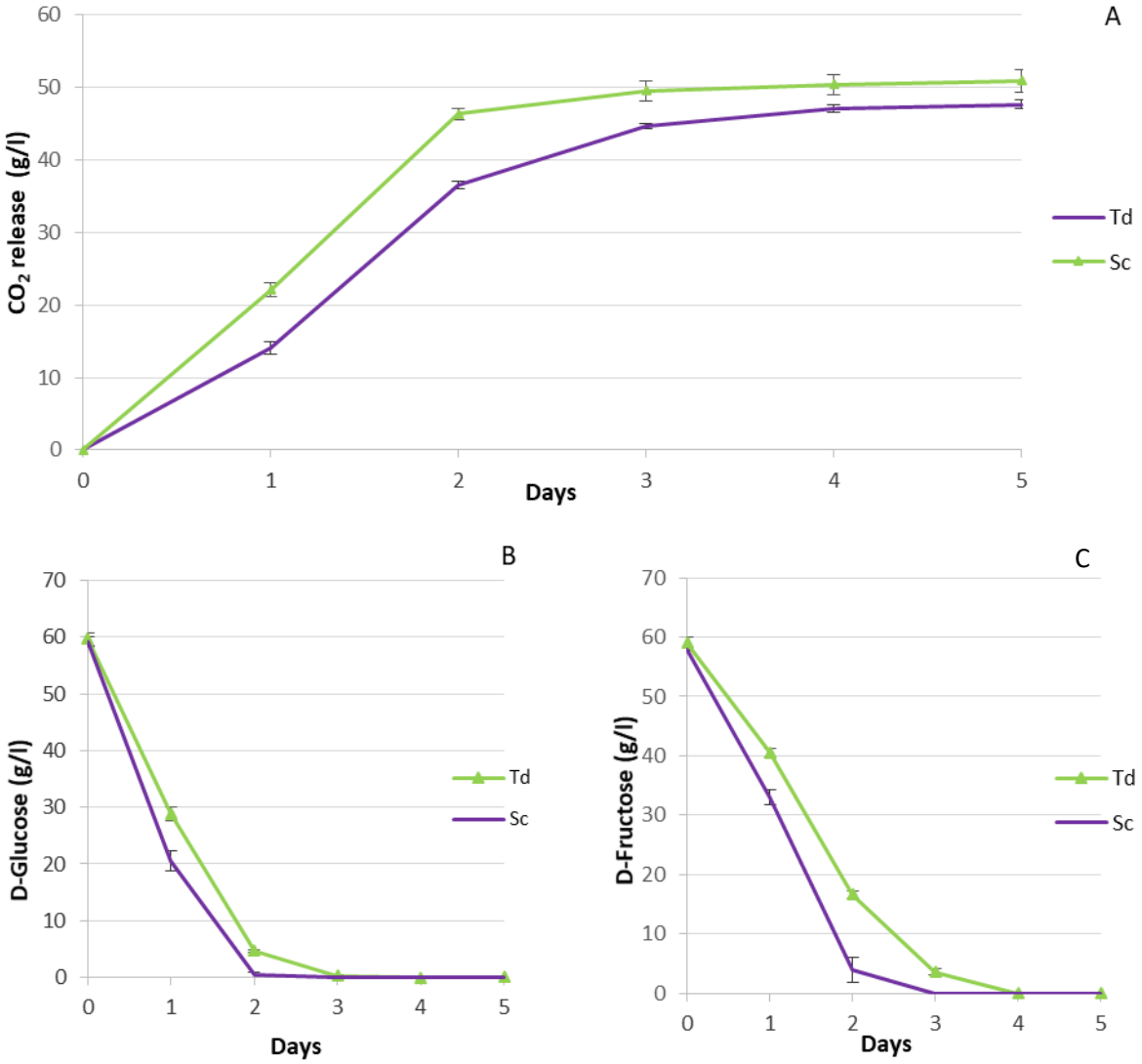
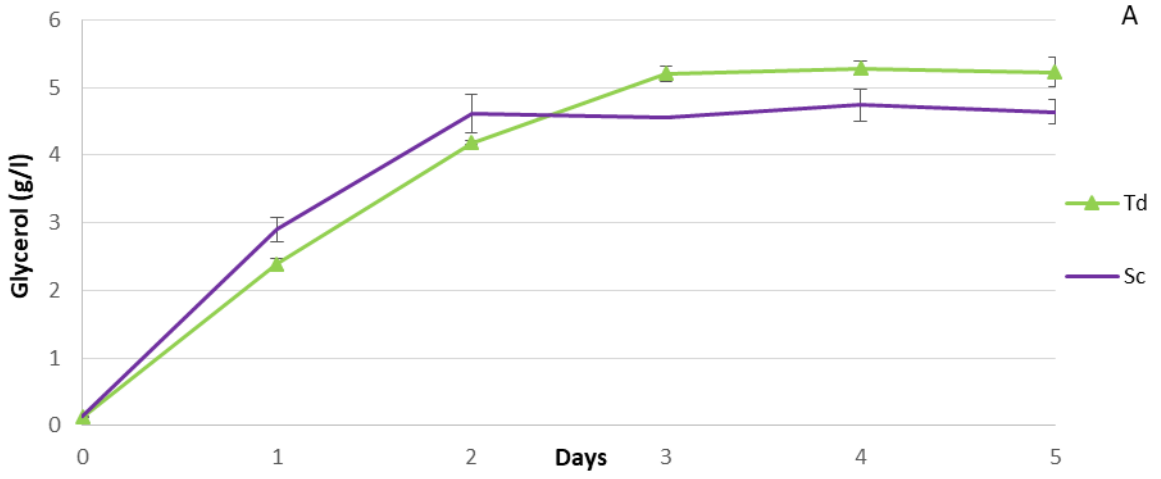


Figure 11: Fermentation kinetics in low sugar must: A) Fermentation rate; B) Glucose consumption; C) Fructose consumption. Td- *T. delbrueckii*, Sc-*S. cerevisiae.*



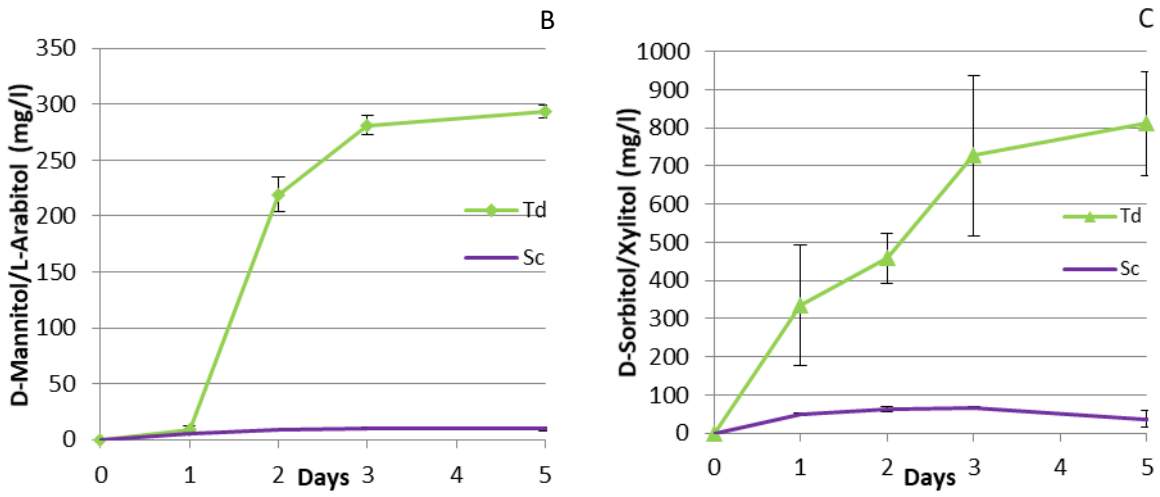


Figure 12: Sugar alcohol production in low sugar must: A) Glycerol; B) D-Mannitol/L-Arabitol; C) D-Sorbitol/xylitol. Td- *T. delbrueckii*, Sc-*S. cerevisiae.*

3.3.4 Polyol production under a variety of environmental conditions

3.3.4.1 Impact of initial salt concentration

The impact of salt on polyol production was investigatd in *T. delbrueckii* CRBO L0544 and *S. cerevisiae*. Similar fermentation rates and growth was observed for these yeasts with the exception of synthetic must containing 0.5 M NaCl. Growth was reduced in this medium (Figure 13 and S3a) and fermentation rate was slower. However, all sugars were consumed in both yeasts by day 3.

In Figure 14a glycerol was increasingly produced from the onset and plateaued by day 3 in all fermentations. The highest amount of glycerol was observed in must supplemented with 0.5 M NaCl in both yeasts with *S. cerevisiae* (6 g/l) producing slightly higher levels of glycerol than *T. delbrueckii*. In contrast, *T. delbrueckii* produced higher levels of glycerol (3.9 g/l) than *S. cerevisiae* (3.4 g/l) in synthetic must supplemented with 0.1 M NaCl. Overall, glycerol levels were similar in fermentations when 0.05 M NaCl and control media were used with levels ranging from 3.6 g/l – 4 g/l.

Mannitol/arabitol was increasingly produced till fermentation end and the highest amount was detected in medium containing 0.5 M NaCl for *T. delbrueckii* (ca. 120 mg/l). Interestingly, more mannitol/arabitol was detected in the control media in comparison to synthetic must supplemented with 0.1 M NaCl and 0.05 M NaCl (Figure 14b). Furthermore, D-mannitol levels were observed to decrease as the salt concentration increased when using GC-MS in *T. delbrueckii* (Figure 15b). Similarly to the previous section, D-arabitol was detected at the highest amounts in all fermentations. NaCl was observed to impact D-arabitol production as increasing

amounts of this polyol were detected in media of increasing salt concentrations for *T. delbrueckii* (Figure 15a).

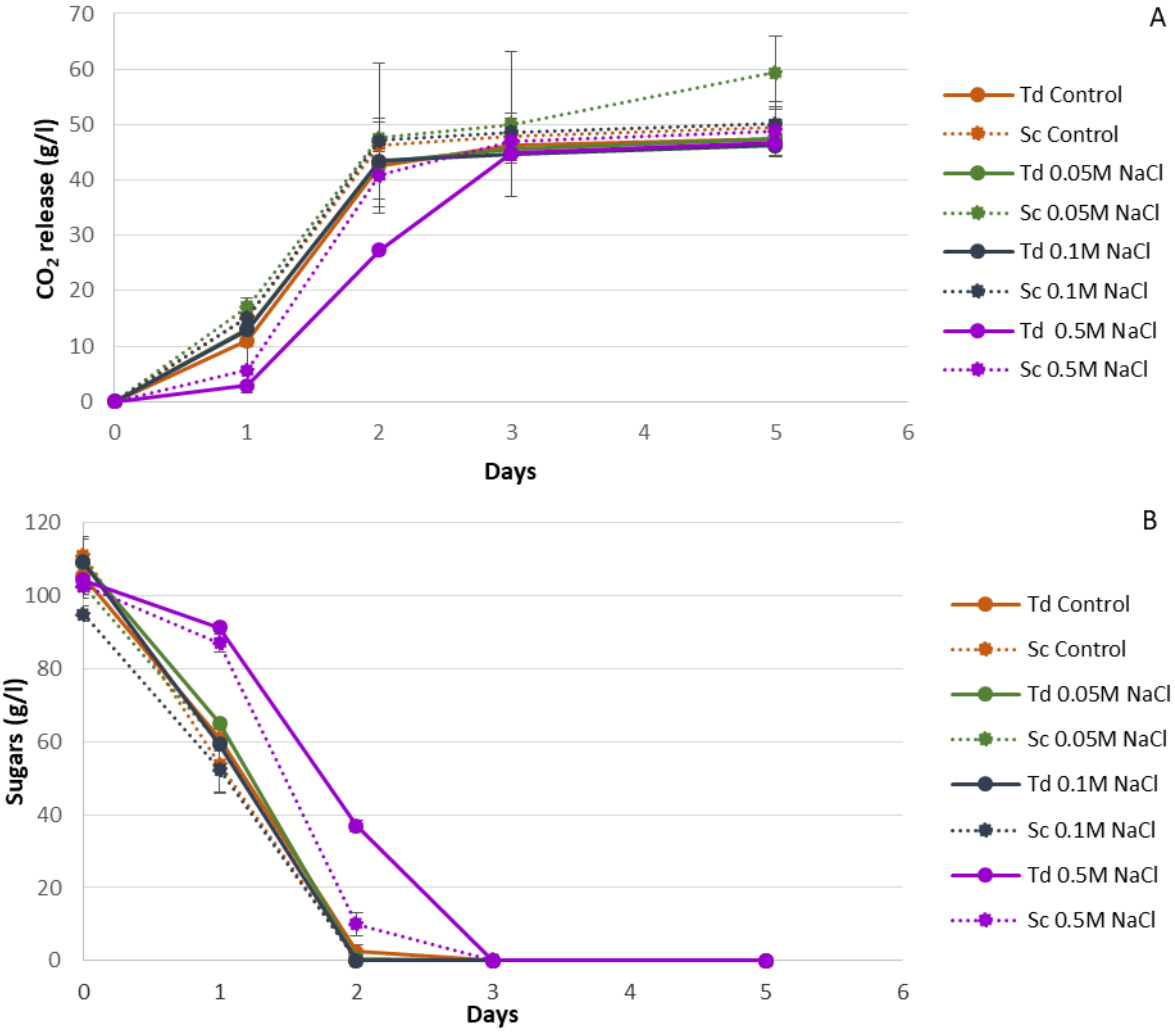


Figure 13: Fermentation kinetics in synthetic must of varying NaCl concentrations: A) Fermentation rate; B) Sugar consumption. Td- *T. delbrueckii*, Sc-*S. cerevisiae.*

When the sorbitol/xylitol assay was used, polyol production was observed to not be influenced by NaCl in *T. delbrueckii* (Figure 14c). This was confirmed via GC-MS analysis as the highest amount of D-sorbitol was observed in the control must and the concentration of this sugar alcohol also decreased with increasing NaCl concentrations (Figure 15c). Furthermore, similarly to previous results (Figure 7c and 8), a discrepancy in the amount of sorbitol detected using enzyme assays versus analyses with GC-MS was observed. However, in this section the difference was much higher. For example in *T. delbrueckii,* 173 mg/l sorbitol was observed in the assay whereas only 19 mg/l D-sorbitol was detected when GC-MS used in must containing 0.5M NaCl (Fig. 15c). Indeed as described in the enzyme assay manual, glycerol may interfere sorbixol/xylitol analyses. Using enzyme assays and GC-MS, *S. cerevisiae* was clearly observed

to produce the lowest amounts of polyols regardless of salt concentration with the exception of glycerol.

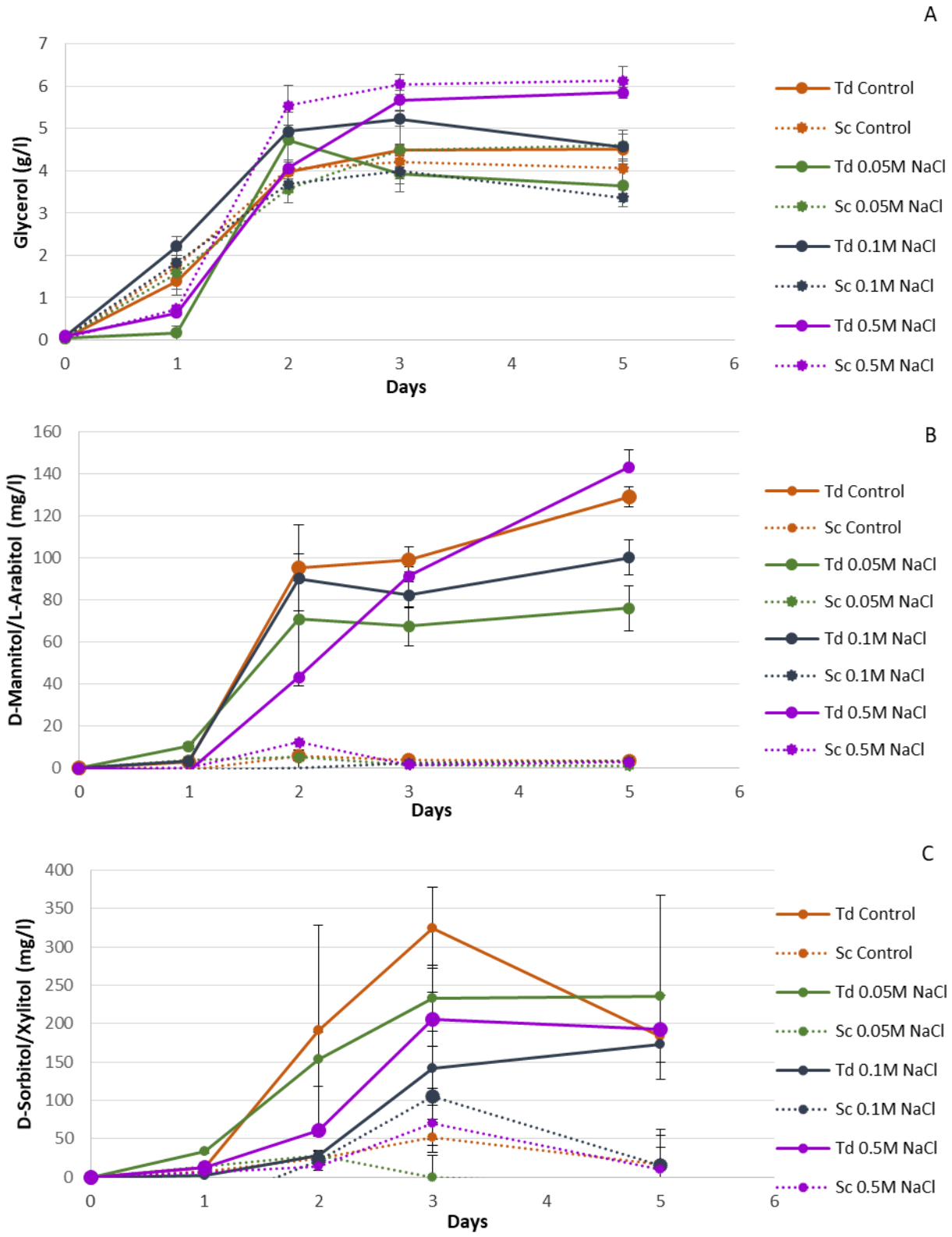


Figure 14: Polyol production during alcoholic fermentation in synthetic musts of varying NaCl concentrations: A) Glycerol; B) D-Mannitol/L-Arabitol; C) D-Sorbitol/xylitol. Td- *T. delbrueckii*, Sc-*S. cerevisiae.*

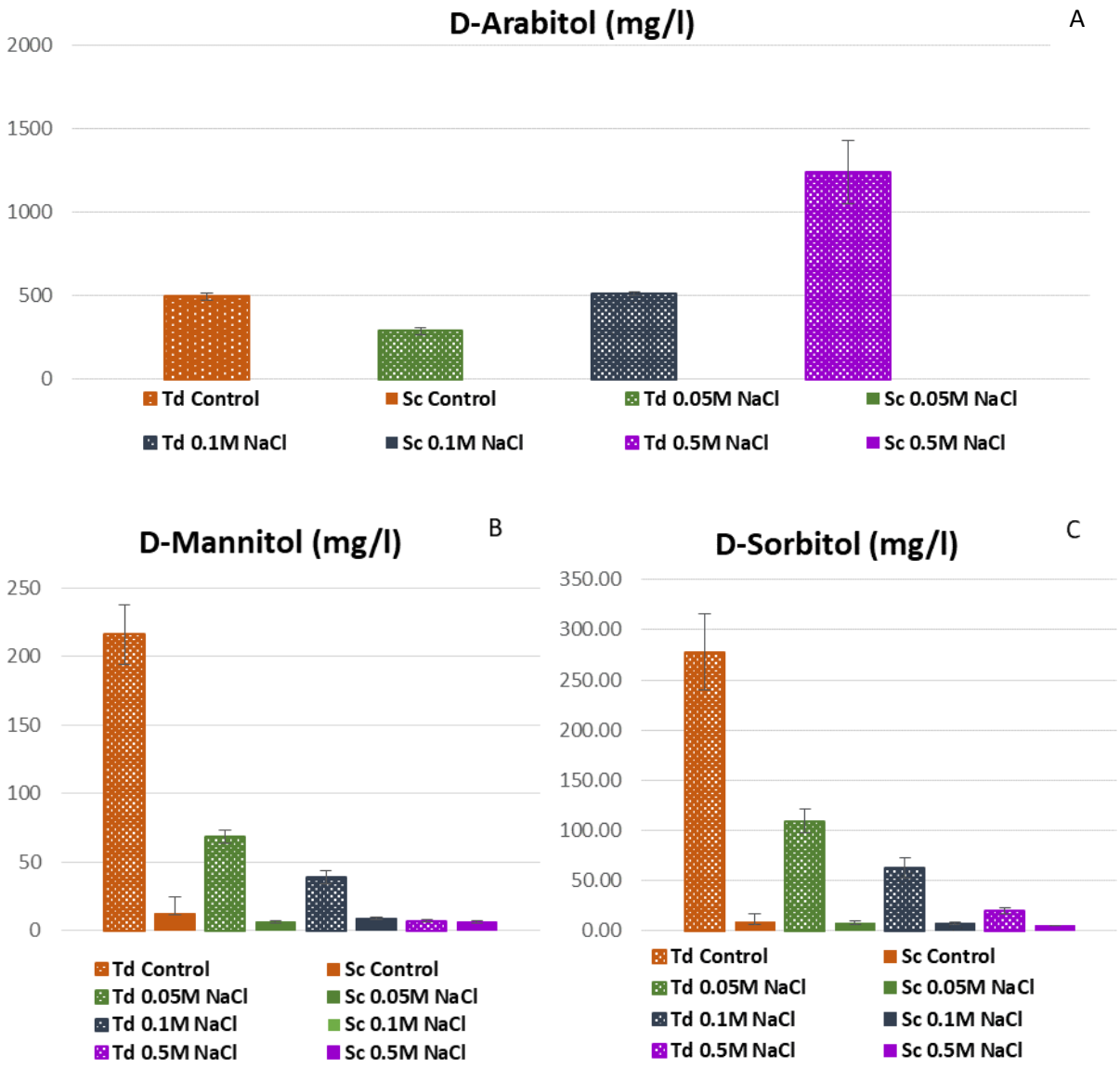


Figure 15: Polyol production at fermentation end in media containing varying amounts of NaCl: A) D-arabitol; B) D-mannitol; C) D-sorbitol Td- *T. delbrueckii*, Sc-*S. cerevisiae.*

3.3.4.2 Minimal media supplemented with nitrogen or lipids

To further understand the impact of environmental conditions on polyol production as well as the possible functions of these compounds, the impact of nutrient supplementation (nitrogen and lipids) on the synthesis of sugar alcohols was tested in minimal media. From Figure 16 and Fig S3b, it is clear that the minimal medium was limiting for *T. delbrueckii*'s growth and fermentation. As a result, a residual sugar concentration of 43 g/l was observed in the control media for this yeast. Although the fermentation rate of *T. delbrueckii* increased when lipids were added to minimal media (10 g/l sugar decrease in comparison to the control), the yeast could still not consume all the sugars (Figure 16b). Fermentation was only complete in amino acid

supplemented media in *T. delbrueckii* whereas *S. cerevisiae* consumed all sugars regardless of the conditions.

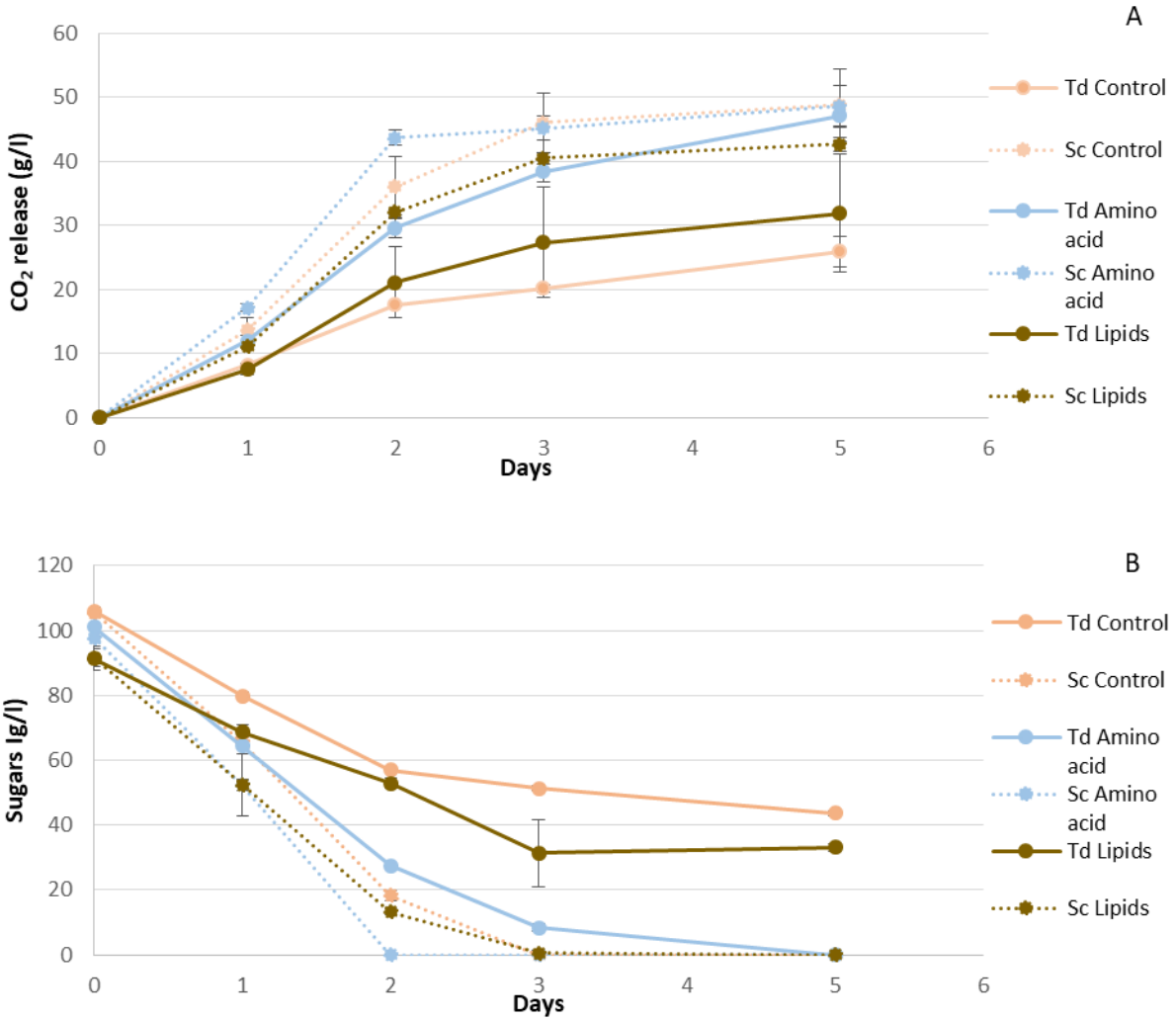


Figure 16: Fermentation kinetics in nutrient limited conditions: A) Fermentation rate; B: Sugar consumption. Td- *T. delbrueckii*, Sc-*S. cerevisiae.*

To determine whether the amount of polyols produced were a result of the type of media used, sugar alcohols were normalized by the percentage of sugar consumed (Figure 17). As observed throughout this study, glycerol was produced at the highest amount. Glycerol was increasingly produced at the early stages and decreased as a consequence of the percent of sugar consumed but as fermentations progressed two groups were observed. *T. delbrueckii* and *S. cerevisiae* in the control media along with *S. cerevisiae* in lipid supplemented media produced the highest amounts of glycerol. *S. cerevisiae* and *T. delbrueckii* in amino acid supplemented media as well as *T. delbrueckii* in lipid containing media produced lower amounts of glycerol.

The highest amount of mannitol/arabitol was detected in *T. delbrueckii* during fermentation in the control followed by amino acid supplemented media. In contrast, the highest amount of sorbitol/xylitol was detected in amino acid supplemented media while similar levels were

detected in the control and lipid supplemented media. Since the GC-MS protocol is limited to only dry fermentation samples, analyses could only be performed in amino acid supplemented media (Figure 18). Although the enzyme assay only detected 172 mg/l mannitol/arabitol (data not shown), almost double this amount was detected using GC-MS for D-mannitol. Furthermore, about 300 mg/l D-arabitol (which was not detected by the enzyme assay) was observed. Unlike the previous sections, only slight differences were observed between the sorbitol/xylitol assay and GC-MS. The amount of sorbitol/xylitol (380 mg/l) detected in the assay differed slightly to the concentration of D-sorbitol (369 mg/l) observed using GC-MS.

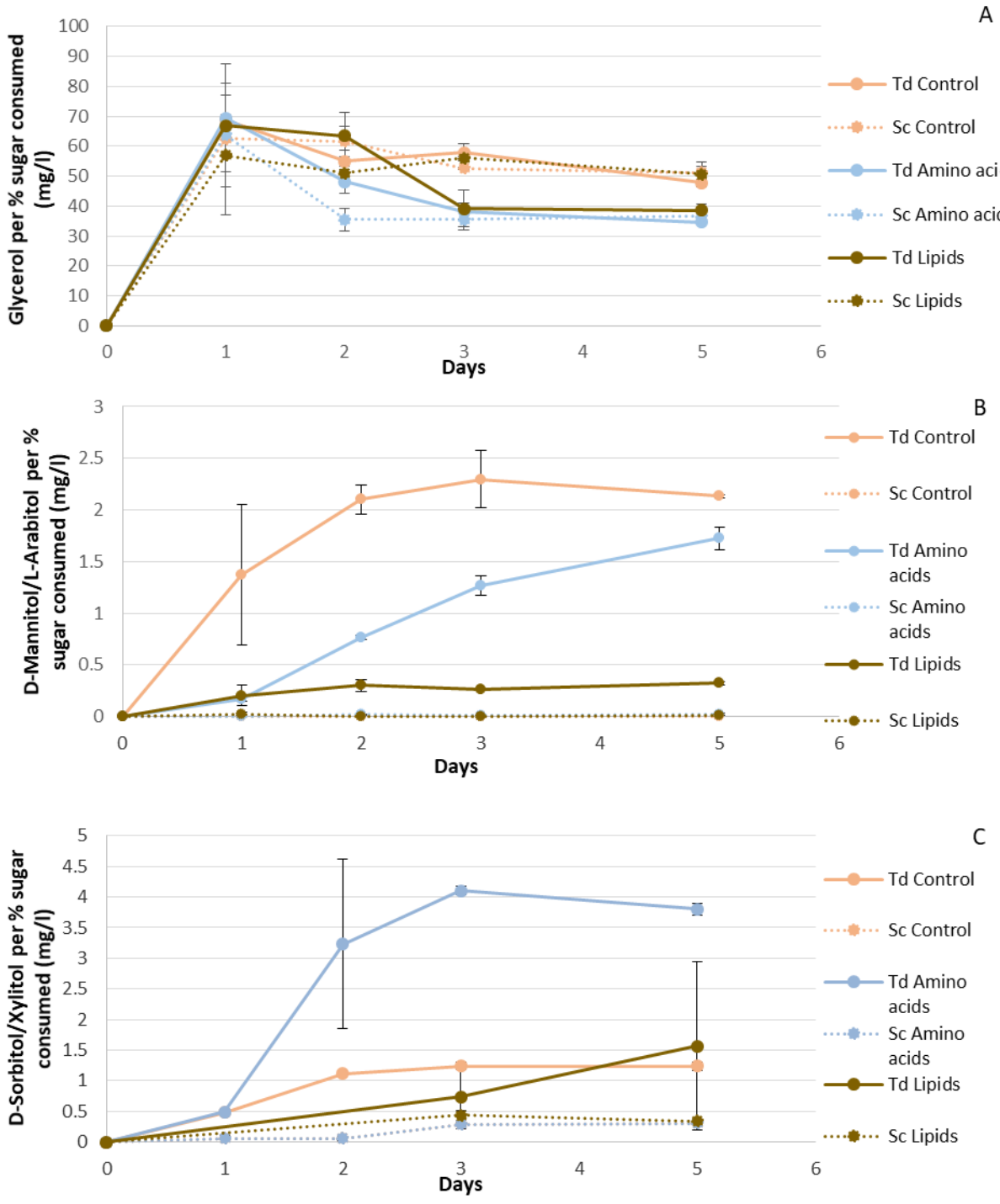


Figure 17: Polyol production during fermentation in minimal media A) Glycerol; B) D-Mannitol/L-Arabitol; C) D-Sorbitol/xylitol. Td- *T. delbrueckii*, Sc-*S. cerevisiae*.

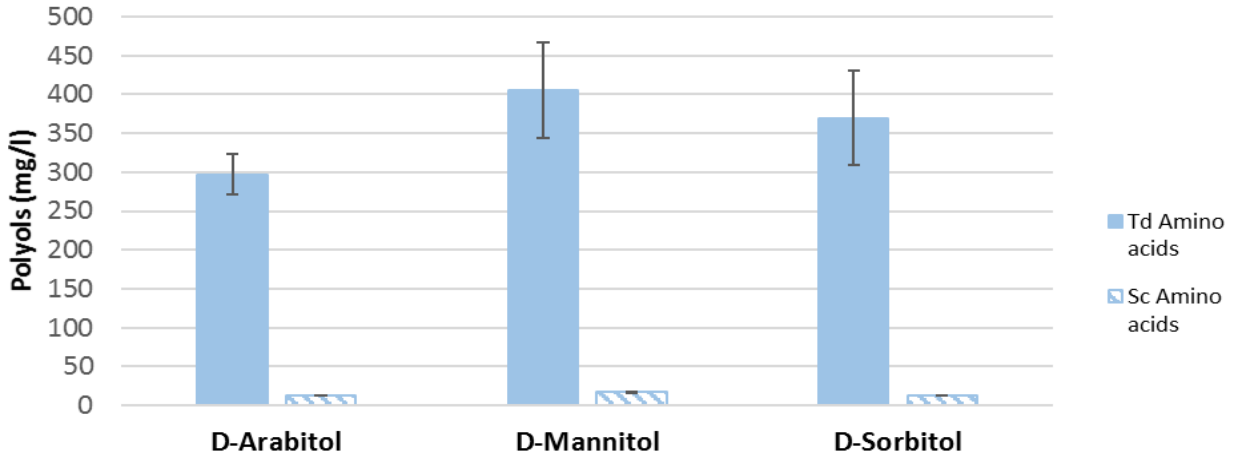


Figure 18: Polyol production at fermentation end in minimal media supplemented with amino acids. Td-*T. delbrueckii*, Sc-*S. cerevisiae*.

3.3.4.3 Acetic acid and ethanol

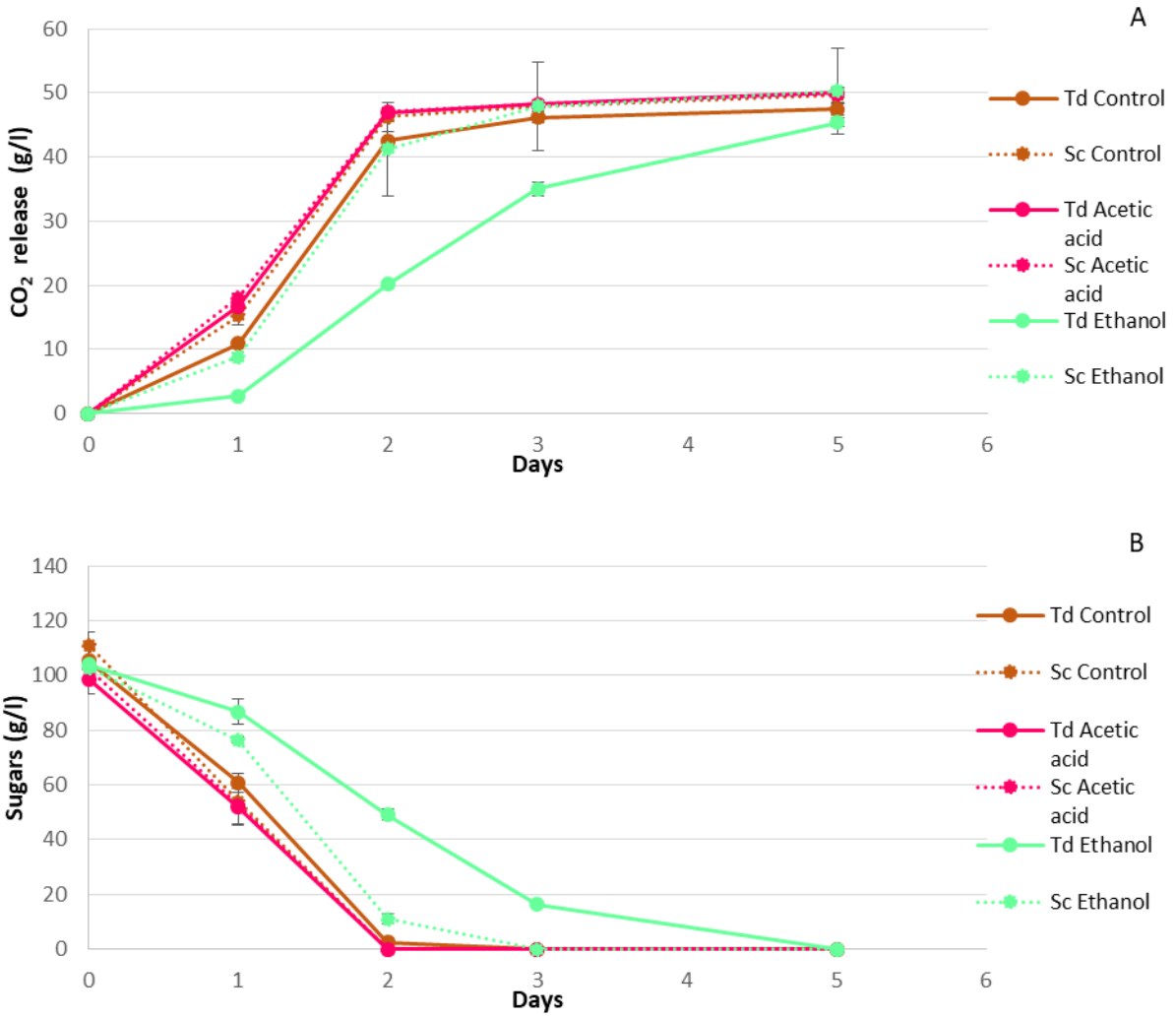


Figure 19: Fermentation kinetics during adaption to ethanol and acetic acid: A) Fermentation rate, B: Sugar consumption. Td- *T. delbrueckii*, Sc-*S. cerevisiae.*

While a variety of environmental conditions were tested for polyol production throughout this study, some of these conditions (e.g. exposure to NaCl) are not wine related. In this section, the impact of ethanol (4%) and acetic acid (120 mg/l) on sugar alcohol production was investigated in synthetic must. Since the amount of acetic acid used was low, no influence on fermentation rate was observed and all sugars were consumed by day 2 in Figure 19. However, a reduction in *T. delbrueckii* growth was observed whereas *S. cerevisiae* proliferated well (Figure S3c). Furthermore, a decrease in acetic acid was observed during the early stages of fermentation in both yeasts (with the lowest amount being detected in *T. delbrueckii*) but by day 2, small amounts of acetic acid were produced and levels plateaued from day 3 till fermentation end (Figure S3d). Ethanol was observed to have a greater influence on the fermentation kinetics in *T. delbrueckii* (Figure 19). Whereas *S. cerevisiae* completed fermentation on day 3, sugars were only completely consumed by day 5 in *T. delbrueckii* when 4% ethanol was present. Under these conditions, *T. delbrueckii* growth was reduced during the early stages but as fermentation progressed this yeast grew slightly better in ethanol containing must in comparison to the control.

For both yeasts in the control and acetic acid supplemented media, glycerol was increasingly produced till day 2 where after it plateaued. In Figure 20, the highest amount of glycerol was observed in the control must in *T. delbrueckii* (ca. 4.5 g/l) followed by *S. cerevisiae* (ca. 4 g/l). Even though the fermentation in ethanol containing must was the slowest, *T. debrueckii* and *S. cerevisiae* produced similar amounts of glycerol (ca. 4 g/l) by fermentation end. Interestingly, the lowest amount of glycerol was observed in the medium supplemented with 120 mg/l acetic acid for *S. cerevisiae* (2.5 g/l) followed by *T. delbrueckii* (3 g/l).

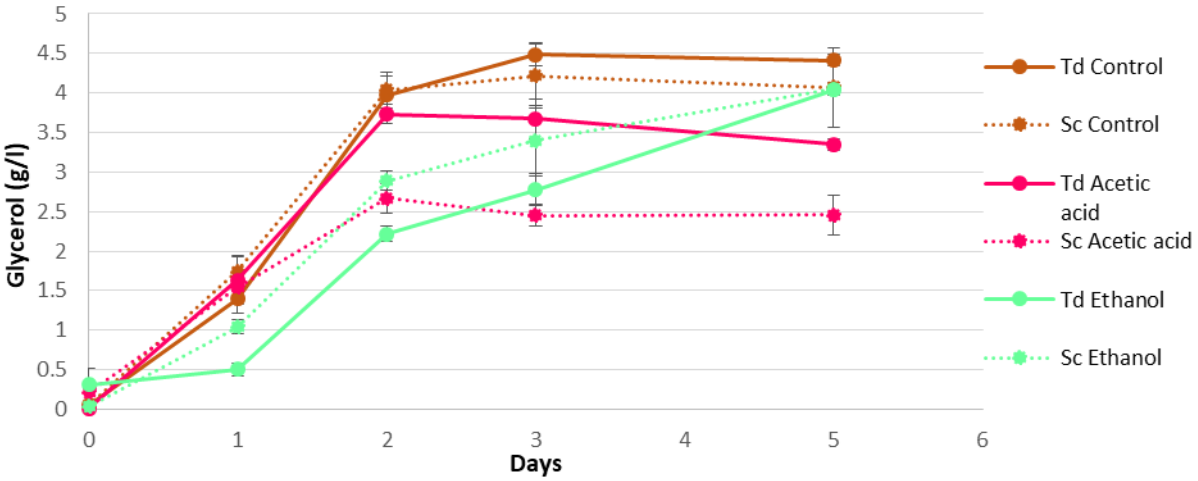


Figure 20: Glycerol production during adaptation to acid and ethanol containing synthetic must. Td- *T. delbrueckii*, Sc-*S. cerevisiae.*

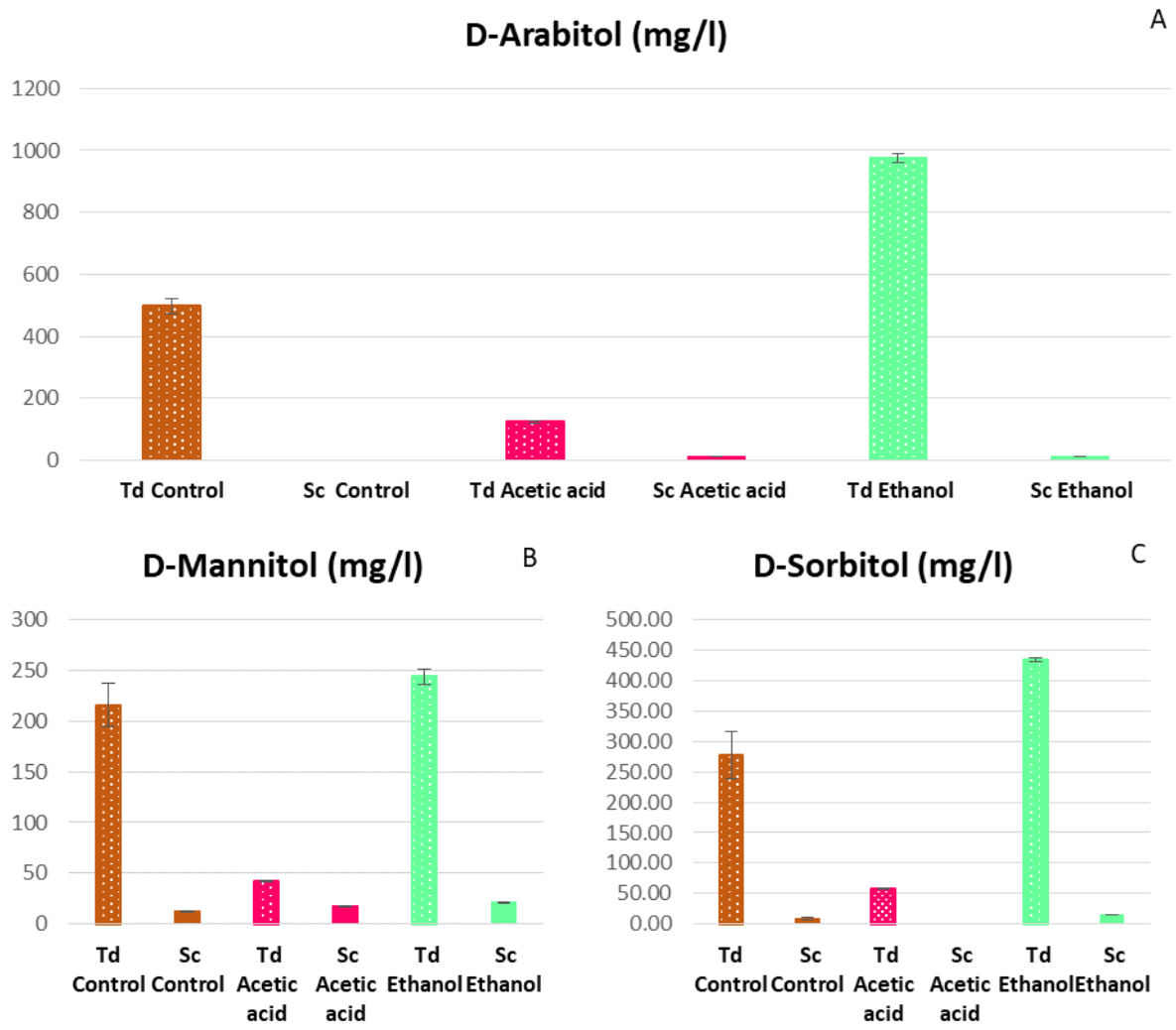


Figure 21: Polyol production at fermentation end in ethanol and acetic acid containing synthetic must: A) D-Arabitol, B) D-Mannitol, C) D-Sorbitol. Td-*T. delbrueckii*, Sc-*S. cerevisiae.*

In comparison to the control (where 496 mg/l D-arabitol was detected), ethanol was observed to influence D-arabitol production in *T. delbrueckii* (976 mg/l) as indicated in Figure 21. The amount of D-mannitol (243 mg/l) in ethanol containing media was slightly higher than what was observed in the control (215 mg/l) in *T. delbrueckii*. The presence of ethanol was observed to impact D-sorbitol production in *T. delbrueckii* - 277 mg/l D-sorbitol was detected in control must whereas 433 mg/l was observed in ethanol containing must. In acetic acid containing medium significantly lower amounts of D-arabitol (ca. 160 mg/l), D-mannitol (ca. 20 mg/l) and D-sorbitol (ca. 50 mg/l) were observed in *T. delbrueckii*. The lowest amounts of these additional polyols was observed in *S. cerevisiae*.

3.4 Discussion

Non-*Saccharomyces* yeasts are becoming increasingly valuable in the winemaking industry due to their peculiar metabolic footprint and, for some of them, their potential to reduce alcohol levels (Capozzi et al. 2015; Medina-Trujillo et al. 2016; Röcker et al. 2016; Wang et al. 2016).

This interesting footprint remains nevertheless ill-characterised at best. Indeed, certain metabolites of potential organoleptic relevance found in wine are still of unknown origin and these include polyols. A range of polyols which may influence wine mouthfeel have been detected in wine but their precise microbial origin has never been unravelled and the concentrations found are usually low (Margalit 2012). However, a recent study showed that certain non-*Saccharomyces* yeasts produce polyols during alcoholic fermentation in synthetic must (De Kock 2015). In this study, the ability of certain non-*Saccharomyces* yeasts to produce polyols during alcoholic fermentation was further investigated and the impact of certain environmental conditions was evaluated.

3.4.1. Separation of polyols using chromatography

An alternative method to polyol enzyme analysis was required as the commercial kits available detect polyols in combination (i.e. D-mannitol with L-arabitol and D-sorbitol with D-xylitol). When standards were set up for chromatography, D-arabitol was calibrated in place of L-arabitol as the latter requires L-arabinose as a substrate (which was not added to synthetic medium) whereas the former can be produced from glucose and fructose (Knoshaug et al. 2009; Kordowska-Wiater 2015). Thin Layer Chromatography (TLC) was unsuccesful for the resolution of polyols in a mixture. Furthermore, smears were observed in *T. delbrueckii* and *S. cerevisiae* samples which could result from silver nitrate binding to proteins and polysaccharides present in grape must and reported to be released by these yeasts (Margalit 2012; Mostert and Divol 2014; González-Royo et al. 2015). Conversely, GC-MS was successful in separating and quantifying polyols of interest, albeit only in fermentation samples with no residual sugars. Samples containing residual sugars can be treated with an Enzytec Glucose Remover or other protocols that by-pass the sugar extraction phase. Throughout the study, enzyme assays were therefore used to monitor the production of polyols during fermentation and GC-MS was used to identify and quantify individual polyols at the end of fermentations in absence of residual sugars.

3.4.2. Fermentation behaviour during yeast screening for polyol production

Three non-*Saccharomyces* yeast species were screened for the synthesis of sugar alcohols in synthetic must containing 230 g/l sugars. These yeasts were initially selected on the basis of their glycerol:acetic acid ratio that seemed to differ from that typically obtained from a *S. cerevisiae* fermentation, as reported in literature (Vilela-Moura et al. 2008; Canonico et al. 2015; Medina-Trujillo et al. 2016). Indeed, this study confirmed previous reports whereby yeasts such as *S. bacillaris* produced high amounts of glycerol and others such as *L. thermotolerans* and *T. delbrueckii* produced lower amounts of acetic acid in comparison to *S. cerevisiae* (Soden et al. 2000; Bely et al. 2005; Englezos et al. 2015; Wang et al. 2016).

Some of the yeasts studied produced other polyols in addition to glycerol, thereby confirming a previous preliminary study (De Kock 2015). *T. delbrueckii* produced the highest amounts of these polyols followed by *L. thermotolerans. St. bacillaris* and *S. cerevisiae*. Unlike sorbitol which was only released into the medium, mannitol/arabitol was initially released but taken back up towards the end of fermentation. This correlated to the time when *L. thermotolerans* and *T. delbrueckii* populations declined which suggests that these compounds may be required intracellularly in unfavourable conditions. In contrast to *S. cerevisiae*, *T. delbrueckii* strains in their anamorphic forms (*Candida colliculosa*) were indeed observed to use sugar alcohols such as sorbitol and mannitol as the sole source of carbon (Costenoble et al. 2003; van Breda et al. 2013).

When GC-MS was used to analyse the end-of-fermentation samples, the three *T.delbrueckii* strains were observed to produce similar amounts of D-mannitol, D-arabitol and D-sorbitol in synthetic medium. Thus, polyol production could be a characteristic of *T. delbrueckii* as a species and not strain dependent. However, other strains should be investigated to confirm this hypothesis. No xylitol was detected in this study possibly because it requires xylose as a substrate-which was not added to the synthetic medium used (Guo et al. 2006). Furthermore, no ribitol was detected in this study during alcoholic fermentation. Studies focused on ribitol production in yeast are limited and the absence of this polyol may be because the metabolic flux is directed towards D-arabitol production instead of ribitol (Ōnishi and Suzuki 1966; Toivari et al. 2010). Indeed, D-arabitol which results from the PPP was produced at the highest amounts (ca. 450 mg/l) in *T. delbrueckii*. This poly has been observed in *Zygosaccharomyces rouxii*, *Candida albicans*, *C. famata*, *Metschnikowia reukaufi* along with *Pichia sorbitophilia* with functions related to osmotic adjusment and redox balance (Wong et al. 1995; Ahmed 2001; Kayingo et al. 2002; Nozaki et al. 2003; Kayingo and Wong 2005). Since *T. delbrueckii* produced similar amounts of glycerol as *S. cerevisiae*, it is possible that the role of additional polyols (in smaller amounts) is related to a higher need for redox balance as opposed to osmoregulation in this yeast under the conditions tested.

3.4.3. Impact of sugar on polyol production in *T. delbrueckii*

Unlike in *T. delbrueckii,* glycerol and acetic acid productions were well correlated to the amount of sugar present or consumed in synthetic must in *S. cerevisiae* as previously observed (Renault et al. 2009; van Breda et al. 2013). However, the amount of D-sorbitol, D-mannitol and D-arabitol was observed to be proportional to the concentration of sugar in *T. delbrueckii* L0544. This is overall in agreement with literature whereby in selected yeasts the amount of sugar present was observed to induce the production of C-3 (glycerol), C-5 (arabitol) and C-6 (mannitol and sorbitol) polyols (Onishi and Suzuki 1968; Dakal et al. 2014) although this did not apply for glycerol in the case of *T. delbrueckii* in this study. Whereas glycerol is limited to recycling NAD^+, the production of the other polyols may allow the recycling of both NAD^+ and

$NADP^+$. This may improve the cell's regulation to redox imbalances (Song et al. 2002; Nozaki et al. 2003; Voegele et al. 2005; Lin et al. 2010). Furthermore, since D-mannitol, D-sorbitol and D-arabitol were produced as glycerol plateued, it is possible that these compounds may replace glycerol for osmotic adjustment/and redox balance as synthesis slows down in *T. delbrueckii*. Similarly in a previous study, glycerol was observed as the main polyol in *Hansenula anomala* while arabitol was produced at a later stage of growth (as glycerol decreased) and it was suggested that arabitol may be produced as an additional compatible solute responsible for yeast viability and as a protectant from sudden stress (Van Eck et al. 1989). *S. cerevisiae* produced low amounts of additional polyols regardless of the initial sugar concentration. However in *gpdΔ* mutants of *S. cerevisiae*, mannitol and sorbitol were observed to function as compatible solutes but the protective effects could not substitute those of glycerol (Chaturvedi et al. 1997; Shen et al. 1999).

3.4.4. Impact of salt on polyol production

In this study, glycerol was the main polyol induced in high salt conditions (0.5 M NaCl) in *T. delbrueckii* and *S. cerevisiae*, thereby confirming previous observations (Posas et al. 2000; Managbanag and Torzilli 2002; Logothetis et al. 2014). However, D-arabitol production was induced by NaCl in *T. delbrueckii* but not in *S. cerevisiae*. In addition to osmotic stress, this polyol was reported to be influenced by oxidative and acid stress in some non-*Saccharomyces* yeasts (Kayingo and Wong 2005; Tomaszewska et al. 2012).

In contrast, D-mannitol and D-sorbitol were observed to decrease with increasing NaCl concentrations in *T. delbrueckii*. Since extracellular amounts of polyols were measured it is possible that in response to the osmotic gradient, mannitol and sorbitol accumulated within the cell with increasing salt concentrations. Indeed in *S. cerevisiae gpdΔ* mutants, intracellular amounts of these polyols were observed to increase in the presence of 0.6 M NaCl (Shen et al. 1999). Salt supplementation was also observed to decrease the intra- and extracellular amounts of mannitol in *Yarrowia lipolytica*. However, this polyol still played a role in osmotic adjustment because the ratio of intra- to extracellular D-mannitol increased upon NaCl shock (Tomaszewska et al. 2012).

In addition to osmoregulation, resistance to high NaCl requires mechanisms which prevent the flow of ions into the cell (through Na^+ exchangers or transporters) and buffer against ion toxicity within the cell. It was suggested that the vacuole is responsible for the intracellular sequestration of Na^+ and that polyols in the cytoplasm may aid in the osmotic adjustment of this organelle while preventing the denaturation of proteins (Nass et al. 1997; Ramsay and Gadd 1997; Nadal et al. 1999). Thus, sorbitol and mannitol may have been accumulated in *T. delbrueckii* to protect enzymes or proteins responsible for Na^+ transport while functioning as osmoprotectants for vacuoles that would have accumulated this cation. Indeed, mannitol and

glycerol were observed to increase the stability of lysozyme in the presence of 0.8 M NaCl (Singh and Singh 2003).

3.4.5 Infuence of nutrients, acetic acid and ethanol on polyol production

Lipid and amino acid supplementation did not improve glycerol production which may be because this polyol is an integral part of yeast metabolism, regardless of nutrient availability (Erasmus et al. 2004; Noti et al. 2015; Goold et al. 2017). Unlike mannitol/arabitol, the amount of sorbitol detected increased in amino acid supplemented medium. This confirms reports that polyol production in yeasts is differentially affected by the cultivation conditions (Gírio et al. 2000; Yu et al. 2006; Lee et al. 2007; Kumdam et al. 2013).

Although acetic acid consumption is usually glucose repressed, selected strains of *S. cerevisiae*, *Zygosaccharomyces bailii* and *L. thermotolerans* were observed to consumed this acid in the presence of glucose under aerobic and semi-aerobic conditions (Vilela-Moura et al. 2008; Vilela-Moura et al. 2010b). Similarly in this study, a reduction in acetic acid was observed during the early stages of fermentation in *T. delbrueckii* and *S. cerevisiae* under semi-aerobic conditions resulting in a decrease in polyol production which also coincided with a decline in *T. delbrueckii* growth. In a previous study, a reduction in glycerol was observed in *S. cerevisiae* after acetic acid intake and it was hypothesised that changes in NAD(P)H oxidation may result in a reduced need for this polyol (Vasserot et al. 2010). D-sorbitol, D-mannitol and D-arabitol may have been affected in the same way for *T. delbrueckii*.

Ethanol is known to inhibit yeast growth and fermentation capacity by compromising the plasma membrane. *T. delbrueckii* was strongly affected by the presence of ethanol as a reduction in fermentation rate and sugar consumption was observed. For a yeast to survive high alcohol levels, changes within the plasma membrane (via fatty acids and sterols) are required to prevent the influx of ethanol while preventing osmotic stress. High amounts of D-arabitol, sorbitol and mannitol were detected in *T. delbrueckii* after exposure to 4% ethanol. This could be an indication of ethanol tolerance as such yeasts were reported to retain less carbohydrate or fluids (Pina et al. 2004a; Pina et al. 2004b; Da Silva et al. 2013).

3.5 Conclusions

Non-*Saccharomyces* yeasts were observed to produce other polyols in addition to glycerol during alcoholic fermentation in this study. *T. delbrueckii* produced the highest amounts of these compounds with little strain variability. In *S. cerevisiae*, the amount of sugar present in grape must influenced the concentration of glycerol in contrast to *T. delbrueckii*. However, the amount of sugar influenced the production of D-arabitol, mannitol and sorbitol in *T. delbrueckii*. Apart from being induced by sugar concentrations, these compounds may be required for co-factor recycling and/or osmotic adjustment during alcoholic fermentation. *T. delbrueckii* responded to high intial NaCl concentrations by accumulating and eventually secreting increased amounts of

glycerol and D-arabitol whereas *S. cerevisiae* overproduced glycerol. The data suggest that mannitol and sorbitol may play different roles in high initial salt conditions as reduced amounts were detected extracellularly with increasing NaCl concentrations. We hypothesise that these compounds may be important for osmotic adjustment or protection of enzymes within the cell. The release of polyols was negatively affected by the presence of acetic acid and induced by ethanol in *T. delbrueckii*. Throughout this study, D-arabitol was produced at the highest amounts which might be an indication of the importance of this polyol for adaptation to a variety of environmental conditions. Nonetheless, further investigations are required to determine the accumulation and transport of these polyols in yeasts under unfavourable conditions in order to unravel the actual roles of these compounds in *T. delbrueckii*'s adaptation. From an oenological perspective, the impact of these sugar alcohols (especially D-arabitol) on wine mouthfeel also requires further studies, as the relatively high concentrations of these polyols could play an important role in enhancing the smoothness/softness perception of wines fermented by *T. delbrueckii*.

3.6 References

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3.7 Supplementary data

3.7.1 Optimization of techniques for the separation of polyols

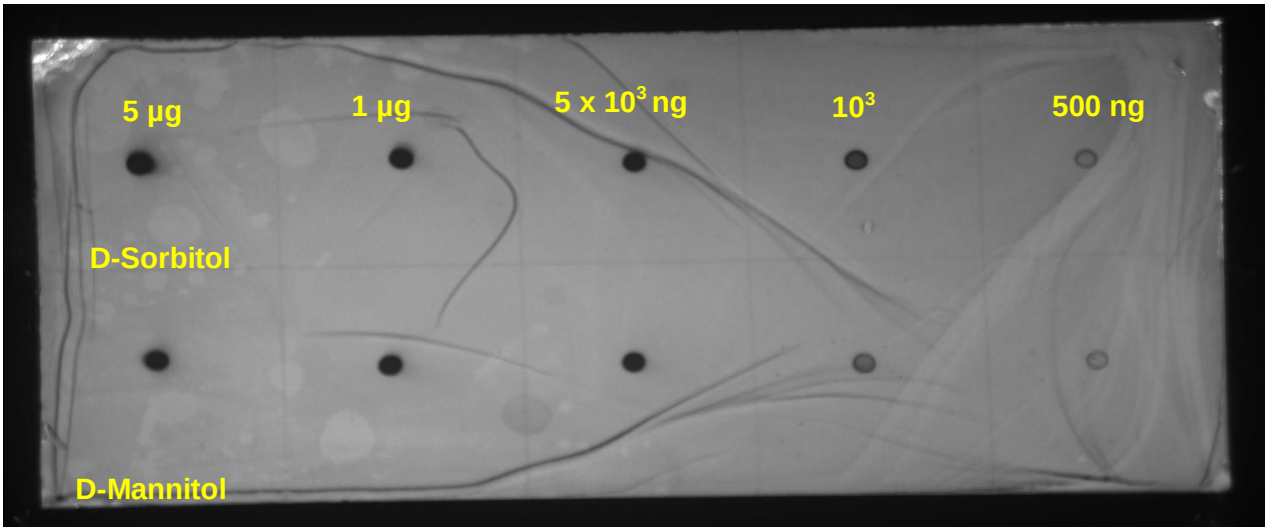
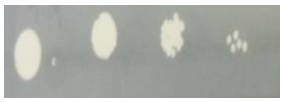


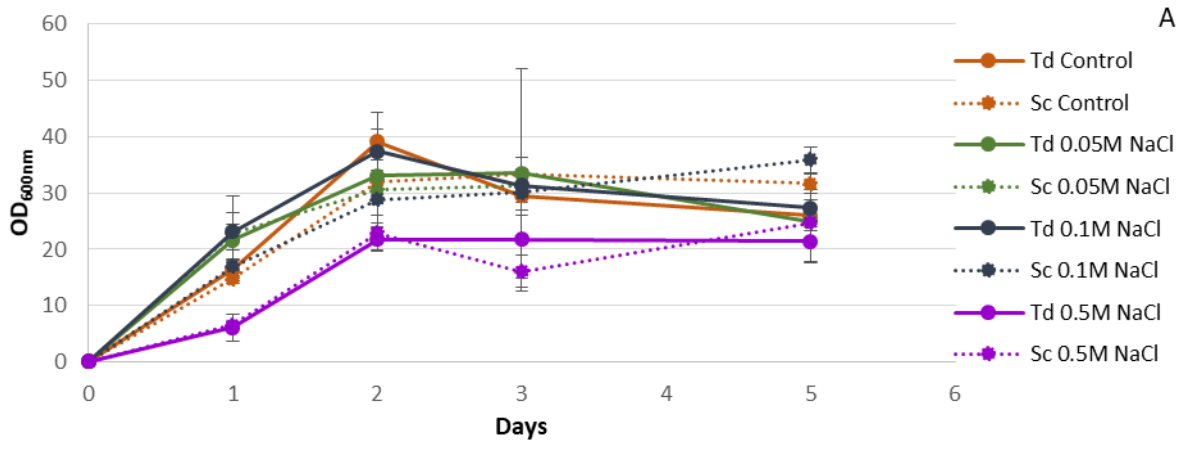
Figure S1: Visualization of D-mannitol and D-Sorbitol at different concentrations with the silver nitrate-sodium thiosulphate dipping system.

3.7.2 Yeast screening for polyol consumption

Table S1: YNB with Glucose, Sorbitol and Mannitol (2 g/l) as carbon sources

Spot plates in Yeast Nutrient Base Agar			
Yeasts	D-Glucose	D-Sorbitol	D-Mannitol
L. thermotolerans			
T. delbrueckii CRBO L0544			
S. bacillaris			
S. cerevisiae			

3.7.3 Yeast growth under different environmental conditions



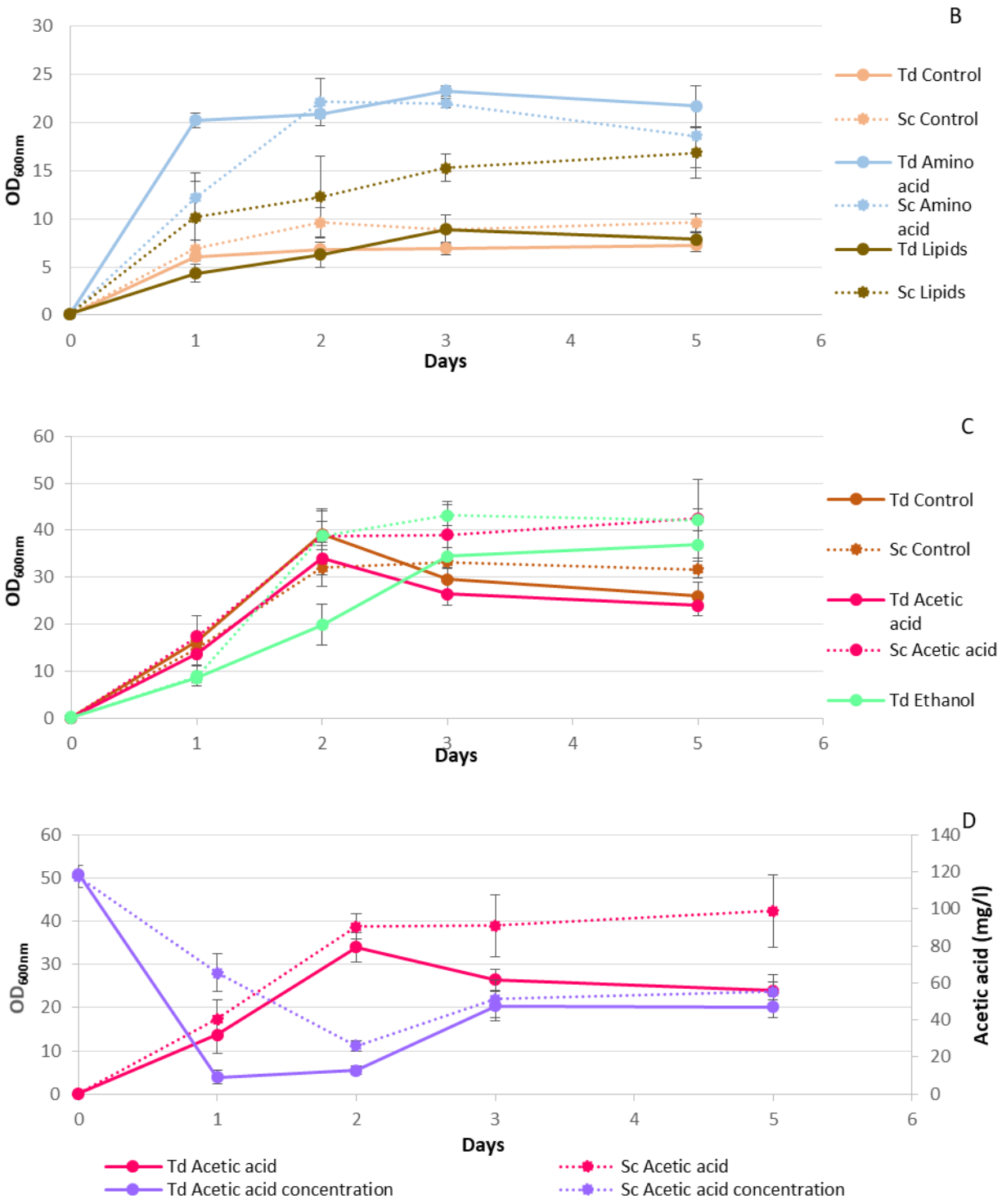


Figure S3: *T. delbrueckii* and *S. cerevisiae* growth under different environmental conditions: A) Yeast growth in synthetic must of different salt concentrations; B) Yeast survival in minimal media supplemented with lipids and amino acids; C) Yeast growth in the presence of acetic acid and ethanol; D) Yeast growth and acetic acid levels in synthetic must supplemented with 120 mg/l acetic acid. Td-*T. delbrueckii*, Sc-*S. cerevisiae.*

Chapter 4

General discussion and conclusions

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4.1 Discussion and conclusions

The use of non-*Saccharomyces* yeasts is becoming increasingly popular in winemaking due to their production of valuable metabolites which might improve wine quality or at least diversify wine styles (Capozzi et al. 2015). The focus of this study was to investigate the production of polyols (which might be important for stress resistance and wine mouthfeel) in non-*Saccharomyces* yeasts of oenological relevance.

Recently, selected non-*Saccharomyces* yeasts were observed to produce polyols in addition to glycerol during alcoholic fermentation. However, polyols were analysed using enzyme assays that detect polyols in combination. Thus one of the objectives of the study was to optimize chromatography based methods for polyol separation. After some optimization, Gas Chromatography-Mass Spectrophotometry (GC-MS) proved successful for polyol analyses in fermentation samples containing no residual sugars.

Similarly to previous studies, glycerol was observed as the main polyol in *Starmerella bacillaris*, *Lachancea thermotolerans* and *Torulaspora delbrueckii* strains (Gobbi et al. 2013; van Breda et al. 2013; Englezos et al. 2015; Wang et al. 2016). However, unlike *Saccharomyces cerevisiae* which produced negligible amounts, these yeasts were also capable of producing other polyols during alcoholic fermentation and the highest amounts were observed in *T .delbrueckii*. Further investigation revealed that the production of fairly high amounts of additional sugar alcohols was a characteristic of the *T. delbrueckii* strains tested. However, more investigations are required to determine whether polyol production is a species or strain dependent trait.

Moreover, *T. delbrueckii* produced similar amounts of glycerol regardless of sugar concentration, as previously observed (Renault et al. 2009; Noti et al. 2015). On the other hand, the production of additional polyols was influenced by the amount of sugar available. The function of these additional polyols may be related to the regulation of redox imbalances during alcoholic fermentation by expanding the co-factor recycling pool or osmoregulation (Shen et al. 1999).

Glycerol and D-arabitol productions were similarly induced in *T. delbrueckii* during adaptation to high amounts of NaCl. Conversely, extracellular amounts mannitol and sorbitol reduced with increasing NaCl concentrations. These compounds may have accumulated and could be required intracellularly for osmotic adjustment (Shen et al. 1999). Furthermore, these polyols may be important within the cell for the protection of enzymes and organelles, thereby allowing normal metabolic activities to continue (Nass et al. 1997; Nadal et al. 1999).

Mannitol/arabitol production was not influenced by nutrient supplementation but sorbitol improved with amino acid supplementation in *T. delbrueckii*. This confirms that polyol production is an intricate process with substrate, co-factor and nutrient requirements specific for the producing organism and sugar alcohol of interest (Gírio et al. 2000; Lee et al. 2007).

In this study, acetic acid supplementation resulted in decreased amounts of polyols. Since small amounts of acetic acid were taken into the cell, it is possible that this could have shifted the NAD(P)H:NAD(P) ratio resulting in a reduced need for glycerol (and possibly the other polyols) to be produced (Vasserot et al. 2010). As ethanol perturbs the plasma membrane's permeability (Pina et al. 2004a), polyols may have been released into the environment as an adaptation mechanism in *T. delbrueckii* during fermentation in ethanol supplemented medium.

From an oenological perspective, the most relevant result of this study is that certain non-*Saccharomyces* yeasts, especially *T. delbrueckii*, were observed to produce other polyols in addition to glycerol and low amounts of acetic acid during alcoholic fermentation. Wine fermented with commercial strains of *T. delbrueckii* have been reported to impart a smooth and rounder mouthfeel to wine (Biodiva^{TM} data sheet, Lallemand). In addition to serving as stress protectants, some of these polyols possess a high relative sweetness in comparison to sucrose (Belitz et al. 2009). Thus, the oenological role of additional sugar alcohols on wine smoothness and mouthfeel needs to be determined.

4.2 Limitations of the study and potential future research

There is a growing interest in the wine industry for the use of non-*Saccharomyces* yeasts due to consumer demands for more complex/diverse wines. However, in addition to a good fermentation capacity, a yeast strain needs to produce valuable metabolites that are not already on market to be considered for commercialisation. Therefore, more non-*Saccharomyces* yeasts that have already been observed to ferment well, produce desirable aromas, metabolites and possess valuable enzymatic activities need to be screened for polyol production to encourage the commercialisation for wine making purposes.

Since non-*Saccharomyces* yeasts are incapable of fermenting to dryness, a GC-MS or High Performance Liquid Chromatography (HPLC) protocol that allows for the detection of individual sugar alcohols in the presence of residual sugars is required. This would allow for the monitoring of individual polyols in non-*Saccharomyces* yeasts throughout alcoholic fermentation in order to further understand the effects of growth, fermentation rate and sugar consumption on the production of these compounds.

The function of polyols should also be investigated in non-*Saccharomyces* yeasts under a variety of environmental conditions. To do this, selected strains can be exposed to heat, oxidative as well as osmotic shock of varying degrees and the total polyol content can be determined. Furthermore, polyols such as erythritol and 2,3-butanediol need to be included during the characterization of sugar alcohol production in non-*Saccharomyces* yeasts in order to determine the total polyol content of wine and whether these compounds serve different roles in conditions of stress. The expression of annotated non-*Saccharomyces* yeasts genomes (such as that of *T. delbrueckii*) could also be investigated under the aforementioned conditions to determine whether polyol production is linked to stress response.

Currently, polyols are produced using a range of substrates with unspecific enzymatic reactions which requires a lot of energy and leads to high amounts of by-products. Thus, there is an interest in the biotechnological production of polyols using yeasts. Wine related non-*Saccharomyces* yeasts can also be investigated for the industrial production of these valuable compounds.

The influence of grape variety on polyol production should be investigated as only one type of grape juice was tested in this study.

Lastly, the impact of sugar alcohols on wine smoothness and mouthfeel should be investigated. Since *T. delbrueckii* produced a range of polyols during alcoholic fermentation, a mixture of these compounds should be used to determine the effects on mouthfeel.

4.3 References

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